Formulation and Stability Studies on Human CuZn Superoxide Dismutase

by

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FORMULATION AND STABILITY STUDIES ON HUMAN CuZn SUPEROXIDE DISMUTASE

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ABSTRACT

Eight formulations of human Cu,Zn superoxide dismutase containing various solvents and buffers were prepared and freeze dried in order to evaluated the stability of superoxide dismutase in formulation. A one year stability study was designed, and the results indicated that all eight formulations had no loss of activity. Two accelerated stability studies were also conducted at 57 °C and 37 °C on four of the formulations containing mannitol and revealed that product #7, which was formulated in a citrate buffer and mannitol, was statistically superior with respect to activity. Stability studies on the four mannitol containing preparations in solution were also performed at room temperature and 4 °C resulting in no statistically significant difference in stability with respect to activity. Biochemical techniques including SDS-polyacrylamide gel electrophoresis, isoelectric focusing, and fast protein liquid chromatography were also utilized and showed that there was no alteration in biochemical properties of superoxide dismutase in formulation throughout the study period. The results obtained in this study indicated that product #7 showed the best overall stability and warrants further investigation.

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Chapter I

INTRODUCTION

1.1. Free Radicals

By definition, a free radical is any species capable of independent existence that contains one or more unpaired electrons in its outer orbital. The unpaired electron(s) gives the molecule special properties. Chemically, unpaired electrons render the molecule extremely reactive, and physically, unpaired electrons make the species paramagnetic(1). The one electron reduction product of oxygen is the superoxide radical. Biologically relevant sources of this radical are autoxidation, and enzymatic oxidation, occurring in subcellular organelles and within intact cells(2). Free radicals are intermediates of oxygen reduction and include superoxide and the hydroperoxyl radical.

1.1.1. Superoxide

When a one electron reduction of oxygen occurs, the product formed is the superoxide radical ($O_2^{\cdot -}$). The electron entering the molecule must place itself into one of the π^* orbitals. The superoxide radical is actually less of a radical than ground state oxygen because it has only one unpaired electron in its outer orbital as compared with ground state oxygen which has 2 lone pair

electrons. Nevertheless, the superoxide radical is much more reactive than ground state oxygen. This is partially due to the spin restriction which has been removed.

The conjugate acid of the superoxide radical is the hydroperoxyl radical ($HO_2\cdot$). The hydroperoxyl radical acts as the supplier of protons. The equilibrium reaction of the conjugate acid/base pair is as follows:

$$HO_2\cdot \longleftrightarrow H^+ + O_2\cdot^-$$

Reaction 1. The Conjugate Acid/Base Pair
Equilibrium Reaction of Superoxide
and The Hydroperoxyl Radical

The pka of $HO_2\cdot$ is 4.88(3). Thus, the amount of superoxide radical will far exceed the hydroperoxyl radical at physiological pH.

The dismutation reaction (Reaction 2), is the most common reaction of the superoxide radical in aqueous solution. The reaction is usually second

$$O_2\cdot^- + O_2\cdot^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

Reaction 2. Dismutation of Superoxide

order. An electron from one superoxide molecule is transferred to another superoxide molecule and the generation of an excessive charge density is avoided by the involvement of a proton. The dismutation reaction has been

thoroughly studied over a wide range of pH values(3,4,5). Figure 1 shows the pH profile of the second order rate constant.

A plateau is observed at approximately pH 2. This corresponds to reaction 3. At a pH of 4.88, equivalent to the pka, the reaction proceeds at maximum

$$2HO_2\cdot \longrightarrow H_2O_2 + O_2$$

Reaction 3. The Hydroperoxyl Reaction at pH 2

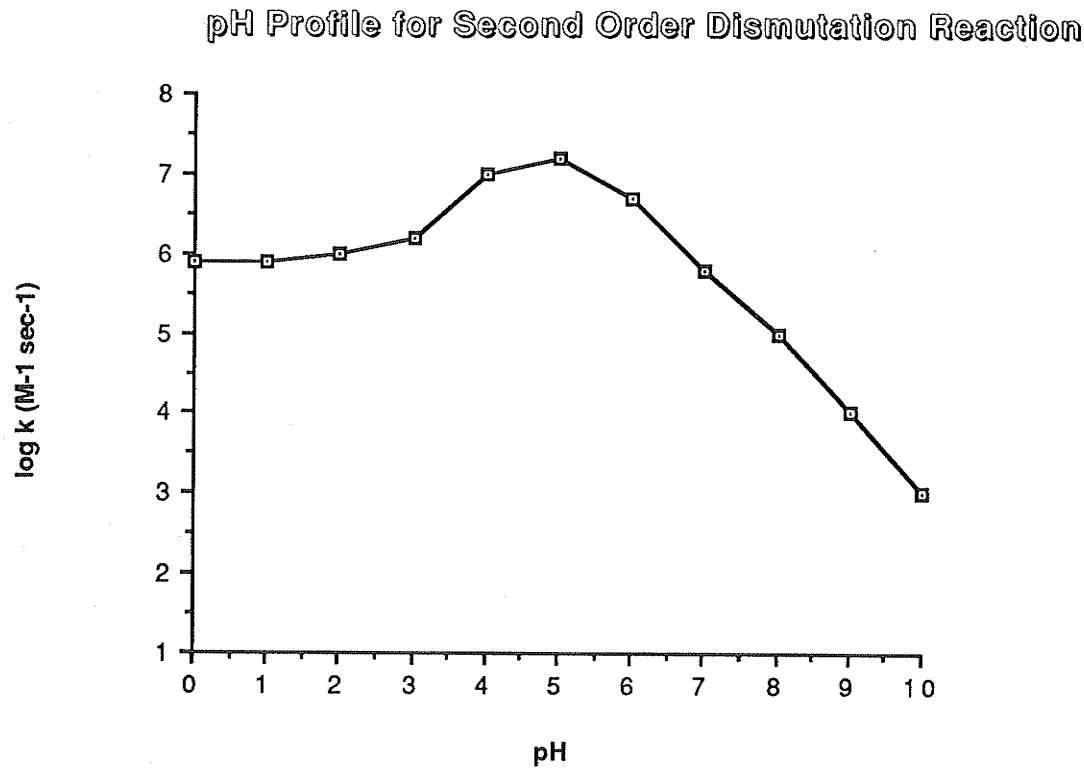


Figure 1. pH Rate Profile For The Second Order Dismutaton Reaction

velocity. This can be written as shown in reaction 4. When the pH is increased beyond 5, there is approximately a 10 fold decrease in

$$HO_2\cdot + O_2\cdot^- \xrightarrow{H^+} O_2 + H_2O_2$$

Reaction 4. Reaction at Maximum Velocity (pH Approximately 5) Between Hydroperoxyl Radicals and Superoxide

the reaction rate per 1 unit change in pH. This region of the graph depicts the reaction involving the addition of a proton into the transitional state(5).

At very high pH values, it is difficult to determine the second order rate constant. Divisek and Kastening(6) reported a value of 6 M^{-1} sec^{-1}. Generally accepted second order rate constants are listed in table 1.

Table 1. Rate Constants of The Dismutation Reaction

REACTION	K (M^{-1} SEC^{-1})
$HO_2\cdot + HO_2\cdot$	7.6×10^5
$O_2\cdot^- + HO_2\cdot$	1.5×10^7
$O_2\cdot^- + O_2\cdot^-$	6

1.1.2. Hydroxyl Radical

The hydroxyl radical reacts with almost every type of molecule found in living cells with extremely high rate constants(7). The reactions of the hydroxyl radical have been classified into three main types, that is, hydrogen abstraction, addition, and electron transfer(8).

Hydrogen abstraction occurs when the hydroxyl radical takes away or "pulls off" a hydrogen atom and combines with it to form water. An example of this would be methanol (Reaction 5). As a result, one of the carbon

$$CH_3OH + OH\cdot \longrightarrow \cdot CH_2OH + H_2O$$

Reaction 5. Hydrogen Abstraction

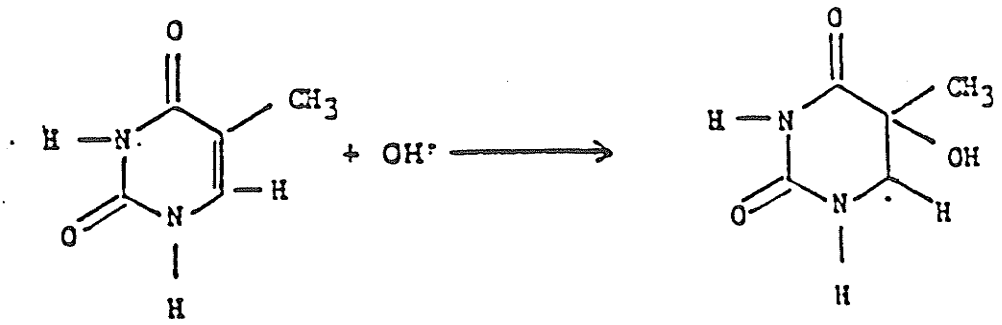
atoms of methanol has been left with an unpaired electron and becomes a radical. This newly formed radical can further react with oxygen (Reaction 6). Another possibility of the newly formed radical from

$$\cdot CH_2OH + O_2 \longrightarrow \cdot O_2CH_2OH$$

Reaction 6. Radical Formation

hydrogen abstraction would be to combine with another radical, forming a covalent bond between the two unpaired electrons. The product of this reaction, unlike the previous example, will not be a radical. Hydrogen abstraction also occurs in the process of lipid peroxidation(9,10,11).

The second main type of reaction involving the hydroxyl radical is that of addition. The addition reaction occurs when the hydroxyl radical adds itself onto another molecule with which it reacts. For example, it has been found that the hydroxyl radical can add across a double bond in the pyrimidine base thymine (8) (Reaction 7).



Reaction 7. Addition

The hydroxyl radical is capable of reacting with purine and pyrimidine bases present in DNA and RNA. The product formed in this reaction is itself a radical that will react further. Along with the damage to the purine and pyrimidine bases of DNA, the hydroxyl radical also causes strand breakage(8,12,13). The results are twofold: firstly, if DNA is damaged extensively it will be destroyed, and secondly, if the DNA does survive, mutation is still a possibility. This is one of the main sources of damage caused by free radicals.

The third main reaction of the hydroxyl radical is electron transfer with organic or inorganic compounds (Reaction 8). The hydroxyl radical is

$$Cl^- + OH\cdot \longrightarrow Cl\cdot + OH^-$$

Reaction 8. Electron Transfer

so reactive that, if produced in a biological system, it will react with any biomolecule within its vicinity, producing other radicals which will vary in reactivity(13).

1.2. Production of Superoxide in a Biological System

1.2.1. Xanthine Oxidase

It has been shown that xanthine oxidase from the liver and probably most other animal tissue is in fact xanthine dehydrogenase which transfers electrons from the substrate to NAD^+ rather than onto oxygen(14). Therefore, hypoxanthine acting as a substrate for xanthine dehydrogenase will not produce superoxide. Xanthine dehydrogenase can be converted to xanthine oxidase through attack by proteolytic enzymes. In view of this, there appears to be some xanthine oxidase activity *in vivo* in the thyroid glands and intestinal cells(14).

Ischemic intestinal cells can convert xanthine dehydrogenase into xanthine oxidase probably as a result of proteolytic attack. Ischemia has many deleterious effects, one being the degradation of ATP and another being the accumulation of hypoxanthine(15). Tissue deprived of oxygen will experience cellular damage. However, it appears that considerably more

damage occurs when oxygen is resupplied(16). This is known as reperfusion damage. This phenomenon is believed to be a result of the xanthine oxidase oxidizing the accumulated hypoxanthine thereby producing excess superoxide molecules that cause the tissue damage (Figure 2). D.A. Parks *et al.* have demonstrated the ability of superoxide dismutase to decrease reperfusion damage(17). With respect to this observation, the administration of superoxide dismutase injected at the right time and under the right conditions, in patients suffering from stroke or heart disease could possibly minimize tissue damage.

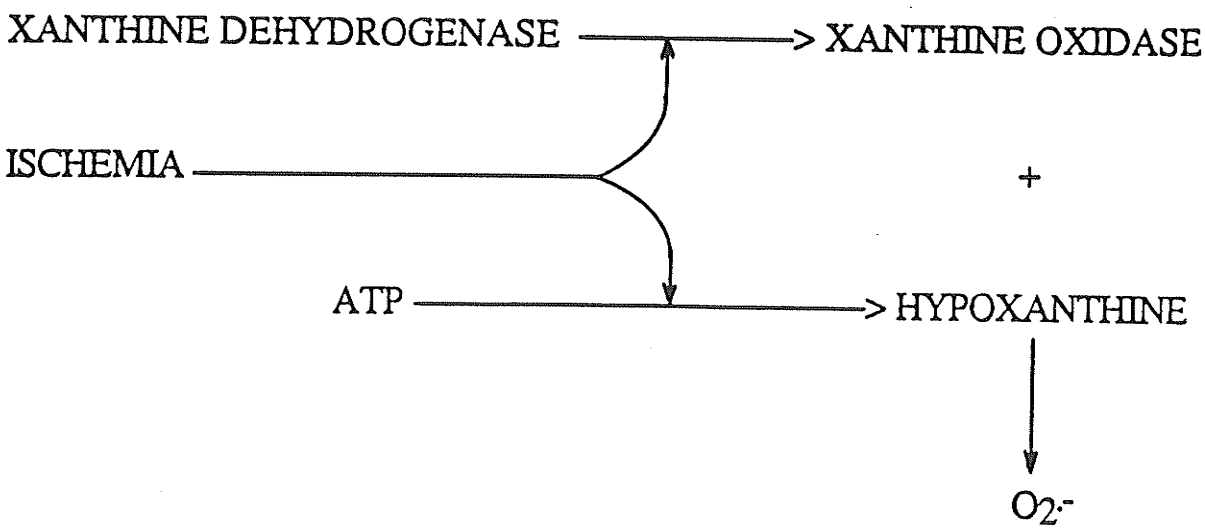


Figure 2. Suggested Mechanism For Damage During Intestinal and Cardiac Ischemia

1.2.2. Oxyhemoglobin

The hemoglobin molecule is composed of 4 protein subunits, each possessing a heme group. Hemoglobin in its deoxygenated state has Fe^{2+} in its heme ring. When oxygen attaches to the iron, an intermediate structure is formed in which the electron is delocalized between the oxygen and iron(19)(Reaction 9).

$$Fe^{2+}\text{--}O_2 \longrightarrow Fe^{3+}\text{--}O_2^-$$

Reaction 9. Electron Delocalization Between Oxygen and Iron

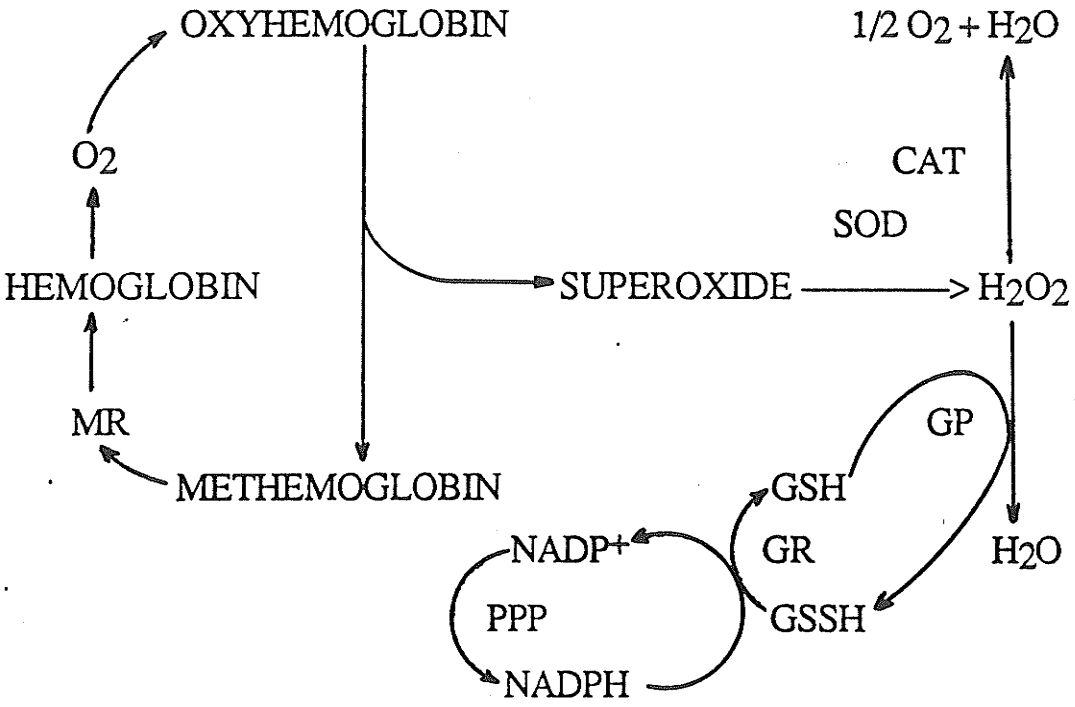
From time to time, oxyhemoglobin will decompose and release superoxide(18). The iron in the heme ring is now in the Fe^{3+} state (Reaction 10).

$$HEME\text{--}FE^{2+}\text{--}O_2 \longrightarrow O_2^{\cdot -} + HEME\text{--}Fe^{3+}$$

Reaction 10. Decomposition of Oxyhemoglobin

In this state (HEME-Fe^{3+}), oxygen is unable to bind. This Fe^{3+} form of hemoglobin is known as methemoglobin. Approximately 3% of hemoglobin undergoes this oxidation reaction and thus erythrocytes are constantly exposed to an environment containing superoxide radicals(19). Since erythrocytes survive on average 120 days, they must make use of defense mechanisms against superoxide radicals. Enzymes such as superoxide

dismutase, catalase, glutathione peroxidase, and those of the pentose phosphate pathway are sources of protection. Along with these protective enzymes, a methemoglobin reductase is present to reduce Fe^{3+} heme to Fe^{2+} heme and restore hemoglobin (Figure 3).



MR-METHEMOGLOBIN REDUCTASE

SOD-SUPEROXIDE DISMUTASE (Zn-Cu)

CAT-CATALASE

GP-GLUTATHIONE PEROXIDASE

GR-GLUTATHIONE REDUCTASE

PPP-PENTOSE PHOSPHATE PATHWAY

Figure 3 Protection Scheme of Erythrocytes Against Damage Resulting From The Oxidation of Hemoglobin

1.2.3. The Electron Transport System

One of the most abundant sources of superoxide radical formation is associated with the electron transport system of the mitochondria(20). Cytochrome oxidase of the electron transport system does not release any detectable oxygen radicals, but instead all radical intermediates are tightly bound to the cytochrome oxidase complex. However, there are other areas of the electron transport system that "leak" electrons. Two areas that have been identified are the NADH-coenzyme Q reductase complex and the reduced forms of coenzyme Q itself(20). This "leakage" of electrons will reduce molecular oxygen to form superoxide. Isolated mitochondria have been shown to produce hydrogen peroxide. This can be attributed to the dismutation of superoxide by superoxide dismutase present in the mitochondria. Magnesium-containing superoxide dismutase has been found in the matrix of the mitochondria whereas copper-zinc containing superoxide dismutase may be found in the space between the inner and outer membranes of the mitochondria(21). In an atmosphere containing 85% oxygen, it is believed that 18% of oxygen uptake is transformed into superoxide radicals(14).

1.2.4. Cytochrome P_{450}

The endoplasmic reticula of many animals contain a special cytochrome known as cytochrome P_{450}. Cytochrome P_{450} oxidizes many substances at the expense of molecular oxygen(22). One atom of oxygen enters the substrate molecule while the other atom is involved in the formation of H_2O.

For cytochrome P450 to function it requires a reducing agent. Reaction 11 is an example of a cytochrome P450 catalyzed reaction. Frequently, the initial

$$AH + O_2 + RH_2 \longrightarrow A{\cdot}OH + R + H_2O$$

Reaction 11. Cytochrome P450 Catalyzed Reaction

product formed will undergo further reactions. Amphetamines, as an example, will become deaminated.

Cytochrome P450 in the liver receives its electrons via a flavoprotein enzyme NADPH-cytochrome P450 reductase(22,23). The reduced form of NADPH-cytochrome P450 reductase is capable of reducing molecular oxygen to superoxide. Isolated microsomal fractions from various tissues have demonstrated the production of hydrogen peroxide in the presence of NADPH presumably due to the dismutation of superoxide(24).

1.2.5. Cytochrome b5

Liver endoplasmic reticulum contains the desaturase enzyme system. This causes formation of C-C double bonds in fatty acids(22). Requirements of this system include oxygen, NAD(P)H, and cytochrome b5. A flavoprotein acts as the electron carrier and transfers electrons from NAD(P)H to cytochrome b5 which in turn donates electrons to the desaturase enzyme(22). Both the flavoprotein and cytochrome b5 are capable of reducing oxygen to produce superoxide.

1.2.6. Phagocytosis

Phagocytosis is the process in which a cell is capable of ingesting microorganisms, other cells, or foreign particles. Once the microorganism has been engulfed by the phagocytic cell, various cytoplasmic granules fuse to the phagosomal membrane and allow their contents to mix with the contents of the phagosome. The particle (microorganism or cell) within the phagosome is killed and if possible digested. Eventually the end products of digestion will be expelled from the cell.

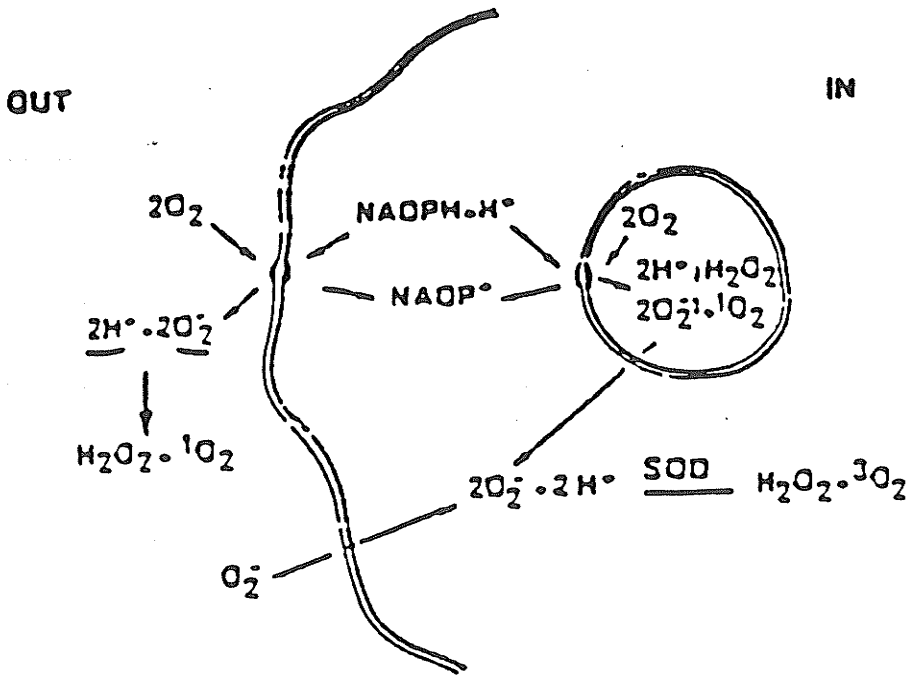


Figure 4. A Model For The Localization of The Superoxide Generating System in Human Polymorphonuclear Leucocytes

An increase of oxygen consumption is observed in neutrophils and macrophages at the onset of phagocytosis. This phenomenon, along with increased anaerobic glycolysis and the generation of superoxide and other oxy radicals are known as the respiratory burst(25). The activation of the respiratory burst depends upon some form of disturbance of the membrane, either by a particle or perhaps certain chemicals. The respiratory burst can occur in the absence of phagocytosis(25). The respiratory burst is also responsible for the generation of superoxide radicals (Figure 4). An enzyme complex associated with the membrane NADPH oxidase is found in the cytosol and is activated by perturbation of the membrane. The electrons from this oxidation reaction reduce oxygen on the outside of the membrane to superoxide. In the case of superoxide formation inside the phagosome, NADPH does not need to cross the phagosomal membrane since the phagosome is surrounded by a piece of inverted plasma membrane(25). As a result, superoxide radicals will be produced within the phagosome where the ingested particle is found.

As already explained, activated neutrophils and macrophages not only release superoxide in phagosomes but also into the extracellular fluid. Once in the extracellular fluid, superoxide can further react to produce hydrogen peroxide and hydroxyl radicals(26,27). These species can attack foreign cells and bacteria in the extracellular fluid. Unfortunately, the extracellular fluid has few protective enzymes such as superoxide dismutase, catalase, and glutathione peroxidase. The deficiency of these enzymes in the extracellular fluid allows superoxide to react further with hydrogen peroxide to produce hydroxyl radicals which in turn can destroy the phagocytes and other cells in

the surrounding area(26). The killing of the phagocytes is a result of lipid peroxidation(14).

1.3. Formation of Hydroxyl Radicals in a Biological System

1.3.1. Ionizing Radiation

Exposure of cells to ionizing X-rays or γ-rays results in hydroxyl radical formation(13). This is attributed to the ionization of H_2O (Reaction 12). Excited water molecules undergo homolytic fission. This homolytic reaction

$$H_2O \longrightarrow H_2O^+ + e^- + H_2O^*$$

H_2O^* - Excited Water Molecule

Reaction 12. Ionization of Water

produces hydrogen atoms and hydroxyl radicals (Reaction 13). The reactions in figures 8 and 9 occur simultaneously and produce hydroxyl radicals.

$$H_2O^* \longrightarrow H\cdot + OH\cdot$$

Reaction 13. Homolytic Reaction

$$H_2O^* + H_2O \longrightarrow H_3O^+ + OH\cdot$$

Reaction 14. Production of Hydroxyl Radicals From Ionizing Radiation

1.3.2. Trace Metals and Hydrogen Peroxide

A feasible source of *in vivo* hydroxyl radical formation is from the reaction of Cu^{+} or Fe^{2+} with hydrogen peroxide, provided the metal ions are available. Approximately 65% of iron in adults is found associated with hemoglobin(28). Myoglobin accounts for another 4% of iron. A small percentage of internal iron is found in various enzymes and transferrin. The remaining iron is present in intracellular ferritin and hemosiderin(28).

Each molecule of transferrin can bind two atoms of Fe^{3+}. Under normal circumstances, transferrin is only about 33% saturated with iron(29). In view of this, it would seem reasonable that the amount of free iron in blood plasma will be virtually zero. It is possible that the intracellular non-protein bound iron could provide iron for the Fenton's reaction and produce hydroxyl radicals(30) (Reaction 15).

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH\cdot + OH^{-}$$

Reaction 15. Fenton's Reaction

These non-protein bound iron atoms are thought to be attached to chelating agents such as ATP, GTP and citrate. These chelates have been shown to react with hydrogen peroxide *in vitro* (31). This source of non-protein bound iron however is very small. Other conceivable sources of iron available for the Fenton's reaction have been located in the cerebrospinal fluid, sweat, and synovial fluid from patients with rheumatoid arthritis(9,31,32). The Fenton's reaction would not be expected to occur in

the plasma since transferrin has great potential to bind iron. However, in the case of an iron overload where transferrin is reaching its capacity of iron binding, free iron will be available for the Fenton's reaction. In fact, the pathology of iron overload may be related to the formation of hydroxyl radicals. Therefore, since iron is available intracellularly and in some extracellular fluids, production of hydroxyl radicals via the Fenton's reaction is possible. Copper on the other hand does not appear to allow formation of hydroxyl radicals.

Copper is absorbed from the stomach or upper small intestine in the form of complexes with aminoacids such as histidine or small peptides(33). When these copper complexes enter the blood most of the copper binds tightly with albumin(33). Copper is also taken up by the liver. Once in the liver, copper is incorporated into ceruloplasmin. When ceruloplasmin is released into the circulation it accounts for approximately 95% of the total copper in the plasma(34). Ceruloplasmin has a strong affinity for copper and will not easily release it. Therefore, copper's ability to stimulate hydroxyl radical formation is inhibited by its strong protein binding. Likewise, the protein concentration within cells is high and free copper, if available, would become protein-bound immediately.

1.3.3. Ethanol Metabolism

Ingested ethanol is metabolized in the liver by alcohol dehydrogenase to acetaldehyde. Ethanol metabolism is also aided by a "microsomal ethanol oxidizing system" (MEOS)(35). The endoplasmic reticulum is the organelle in which the MEOS originates. Also within the endoplasmic reticulum is a

system capable of producing hydrogen peroxide. The hydrogen peroxide allows the generation of hydroxyl radicals which can oxidize some of the ethanol. The generation of hydroxyl radicals in this system may be due to a Fenton reaction using microsomal membrane-bound iron.

1.4. Superoxide Dismutase

1.4.1. Copper-Zinc Superoxide Dismutase

The copper-zinc superoxide dismutase (CuZnSOD), isolated from human eukaryotic cells has a molecular weight of about 32 000 and contains 2 protein subunits(36,37,38,39). Each protein subunit has an active site containing one zinc ion and one copper ion(38,39). Removal of the copper ion results in the loss of enzyme activity, whereas removal of the zinc ion does not result in any loss of activity(39,40). The zinc is not involved in the catalytic function of the enzyme, however it appears to play an important role in the stability of the enzyme. If the copper ion is replaced with another transitional metal such as manganese, activity is lost, whereas the replacement of zinc with cobalt, mercury, or cadmium ion, provides the enzyme with increased stability(14).

Both CuZnSOD and manganese superoxide dismutase catalyze the the dismutation reaction seen previously in reaction 2. This reaction, when catalyzed by superoxide dismutase, is relatively independent of pH in the range between 5.3 and 9.5(5). The rate constant for this enzyme catalyzed reaction is about 1.6×10^9 M^{-1} sec^{-1}. On the other hand, the rate constant for the uncatalyzed reaction is pH dependent and is approximately 5×10^5

M^{-1} sec^{-1} at physiological pH(5). The copper appears to function in the dismutation reaction by undergoing changes in oxidation states (Reaction 16).

$$ENZYME\text{-}Cu^{2+} + O_2^{\cdot -} \longrightarrow ENZYME\text{-}Cu^{+} + O_2$$

$$ENZYME\text{-}Cu^{+} + O_2^{\cdot -} + 2H^{+} \longrightarrow ENZYME\text{-}Cu^{2+} + H_2O_2$$

$$\text{NET REACTION: } O_2^{\cdot -} + O_2^{\cdot -} + 2H^{+} \longrightarrow H_2O_2 + O_2$$

Reaction 16. Changes in Copper's Oxidation State in The Dismutation Reaction

Cyanide is a powerful inhibitor of CuZnSOD(41). Diethyldithiocarbamate will also inhibit the action of CuZnSOD by binding to the copper site and removing it from the enzyme. Almost all of the surface of each protein subunit is negatively charged, and therefore has the tendency to repel the superoxide ion. However, there does exist a positively charged pathway leading to the active site. Thus, superoxide radicals approaching any part of the protein will be guided through the pathway to the active site.

Purification and analytical techniques have shown that there are two slightly different forms of CuZnSOD. The electrophoretic variant of human CuZnSOD is known as SOD-2(42,68). This form of superoxide dismutase has been found in individuals of Northern Sweden and Northern Finland. Most of the population is homozygous for normal CuZnSOD (SOD-1), however, there are a few heterozygotes with both SOD-1 and SOD-2, and only a few homozygotes for SOD-2.

CuZnSOD is found in the cytosol of the cell with some activity found in the lysosomes and possibly between the inner and outer mitochondrial membranes(43).

1.4.2. Human Manganese-Containing Superoxide Dismutase

When human manganese-superoxide dismutase (MnSOD) was first isolated it was found to be quite different from CuZnSOD. The MnSOD was pink as compared with the blue-green colour of CuZnSOD, and MnSOD was found not to be inhibited by either cyanide or diethyldithiocarbamate(44). The rate constants of CuZnSOD and MnSOD at pH 7 are similar, however the rate constant for MnSOD at alkaline pH decreases unlike that of CuZnSOD(5). MnSOD has a molecular weight of approximately 40000 and as the name implies, manganese is found at the active site. In the resting enzyme, manganese is found in the Mn^{3+} state(45). Similar to CuZnSOD, if the metal ion(manganese) is removed from the MnSOD protein, activity is lost.

Subcellular fractionation studies on chicken livers have shown MnSOD to be present in the mitochondrial membranes(45). MnSOD is also found in the matrix of the mitochondria. In human liver, there appears to be some MnSOD outside the mitochondria(45). The production of MnSOD has been observed to increase when copper supply for CuZnSOD is restricted thereby maintaining total cellular SOD activity.

Up to this point, a general overview of the chemistry and biochemistry of superoxide dismutase has been presented. However, for a complete overview of superoxide dismutase, assay systems must be addressed.

1.5. Superoxide Dismutase Assays

Over the years, several assay procedures have been published. It is important to keep in mind when searching for an assay to determine the activity of superoxide dismutase, that the assay must be practical for the experimenter's purposes as well as be readily reproducible. The following section reviews several of the assay systems for superoxide dismutase.

One shortcoming of all superoxide dismutase assays is that saturation of the enzyme with substrate is not approached. Minimal concentration of superoxide at steady state occur in the most commonly used assays. In pulse radiolysis, the rate constant between superoxide dismutase and substrate is measured directly whereas all other assays methods depend on the competition between the enzyme and the detector. As a result, the sensitivity of the assay to superoxide dismutase is determined by the ratio of the rate constants as well as the concentration of the detector. Activity of superoxide dismutase is measured in arbitrary "units". A "unit" is defined as the amount of superoxide dismutase required to inhibit the rate of reduction of the superoxide radical by 50%. Thus, a "unit" is not an absolute amount of enzyme but instead a concentration of superoxide dismutase.

In 1968, McCord and Fridovitch discovered that superoxide dismutase inhibited cytochrome c reduction by xanthine oxidase(46). This discovery led to the first assay for superoxide dismutase. The assay was developed by McCord and Fridovitch and is recognized as the "standard assay" for superoxide dismutase(47). This assay has been modified several times but still remains as one of the most reliable systems.

1.5.1. Generators of Superoxide

Enzymatic

A source of superoxide radicals can be generated by the aerobic action of xanthine oxidase on xanthine. Hypoxanthine and purine can also be used as a substrate if a greater amount or longer duration of radical formation is desired(48). Hypoxanthine produces twice as much superoxide anions per mole as compared to xanthine, whereas, purine produces three times as much. Aliphatic aldehydes have also been employed as a substrate for xanthine oxidase. Aliphatic aldehydes have the added advantage that they do not absorb in the near ultraviolet region of the spectrum. Detectors for enzymatically produced superoxide include cytochrome c, sulfite oxidation, nitroblue tetrazolium, and nitrite formation from hydroxylammonium chloride (47,49,50,51). In the xanthine oxidase/cytochrome c system, the assay suffered from a few shortcomings: (1) The assay was dependent on pure supplies of xanthine oxidase and cytochrome c and was not applicable to acrylamide gels. (2) Interference by agents capable of oxidizing ferrocytochrome c occurred and thus the assay could not be readily applied to crude extracts that might contain cytochrome peroxidase or cytochrome oxidase acitivities(49).

Photochemical

Production of superoxide by the spontaneous re-oxidation of photoreduced flavin was first reported by Massey *et al.*(52). This system made use of cytochrome c as the detector. Two years later Beauchamp and Fridovitch published an assay procedure in which they produced superoxide radicals by the photochemical procedure but instead coupled it with nitroblue tetrazolium to give a spectrophotometric assay(49). The major advantage in Beauchamp and Fridovitch's method is that it can be applied to polyacrylamide and agarose gel electophoresis thereby localizing superoxide dismutase activity bands in gels. The reactants which consists of riboflavin, methionine, and nitroblue tetrazolium may be mixed together under darkened conditions and applied to the gels. Once the gels are removed from the solution the reaction can be started by exposure to light. As superoxide anions are produced photochemically within the gel, the nitroblue tetrazolium is reduced to form an insoluble product known as formazan. In areas where superoxide dismutase is present, the superoxide radicals will be scavenged, thus inhibiting the reduction of nitroblue tetrazolium and as a result, clear zones appear. Excess reactants can be washed out of the gel after color developement, leaving behind insoluble formazan locked in place.

Chemical

Superoxide radicals have been produced via chemical reations and thereby providing the basis for several assays. In 1971, Misra and Fridovitch investigated the autoxidation of epinephrine to adrenochrome(53). They found that this reaction had two possible pathways which are pH dependent.

Superoxide radicals were found to be generated via the autoxidation at a high pH. In the assay procedure, epinephrine is added to a pH 10.2 buffer and the appearance of adrenochrome is monitored at 480 nm. Superoxide dismutase activity can be determined by this sytem since superoxide dismutase inhibits the reaction. When using pure preparations of superoxide dismutase, this assay has proven to be approximately twice as sensitive as the "standard assay" and is convenient for multiple assays. Considerable variability however does occur when working with crude homogenates of tissue. This assay system does provide the added advantage that the superoxide involved in the reaction acts as an oxidant, rather than a reductant. As a result, endogenous reductants may be less of a problem as compared to a system in which superoxide reduces the detector.

Another chemical system used to assay superoxide dismutase is based on the ability of the enzyme to inhibit the autoxidation of pyrgallol(54). With increasing pH the autoxidation of pyrogallol also increases. At pH 7.9 superoxide dismutase inhibits this autoxidation by 99% as compared with 90% inhibition at pH 9.1(54). This illustrates that superoxide plays an important role in the reaction. At higher pH levels however, mechanisms independent of superoxide become favorable. For example at pH 10.6, the autoxidation is inhibited by superoxide dismutase by only only 15%.

Fried reported the production of superoxide anions by aerobic oxidation of NADH, catalyzed by phenazine methosulfate(55). Nitroblue tetrazolium is used as a dectector and is reduced by superoxide to form insoluble formazan. This aerobic reduction of nitroblue tetrazolium in the presence of phenazine methosulfate can be inhibited by superoxide dismutase. This assay is

dependent on the concentration of NADH, and any factors which affects NADH will lead to misinterpretation of the apparent activity of superoxide dismutase. This assay may be used as an "end-point" assay when large numbers of assay must be done.

Electrochemical

In 1961, Fridovitch and Handler reported the generation of superoxide at the surface of a platinum cathode in contact with aqueous solution(56). Electrochemical production of superoxide anions can also be generated in aprotic solvents. This latter method of superoxide generation produces stable and relatively concentrated solutions of superoxide. This solution can be used as a source of superoxide radical and has been used in a system in which it is coupled with tetranitromethane as a detector. Generally speaking, electrochemical production of superoxide is not practical for routine use.

1.5.2. Detectors of Superoxide

Cytochrome c

At pH 7.8 the rate constant for the reaction between ferricytochrome c and superoxide is about 6×10^5 M^{-1} sec^{-1}(5). Since ferricytochrome c is not a specific detector for superoxide many problems may arise. For example, some low molecular weight compounds found in tissue homogenates have the ability to reduce cytochrome. If in large enough quantities, these low molecular weight compounds could interfere with the superoxide dismutase

assay. This problem can usually be alleviated by dialyzing the tissue homogenate.

Cytochrome oxidase can also create problems when assaying tissue extracts via cytochrome c reduction. Cytochrome oxidase re-oxidizes reduced cytochrome c, and as a consequence the results of an assay may be falsely interpreted as increased superoxide dismutase activity since superoxide dismutase inhibits the reduction of cytochrome c.

Problems associated with cytochrome oxidase can be circumvented by adding a low enough concentraton of cyanide not to inhibit the copper-zinc superoxide dismutase. Alternatively, if cytochrome c is heavily acetylated, it is no longer a substrate for cytochrome oxidase. Acetylated cytochrome c will still react with superoxide but the rate constant will be reduced to approximately one-half.

Commercially available products of cytochrome c often contain trace amounts of copper-zinc superoxide dismutase. At a pH of 7.8, this is without significant effect. Unfortunately however, at pH 10, the rate constant is a reduced to about 6×10^4 M^{-1} sec^{-1} for the reduction of cytochrome c by superoxide(5). If 1 mM of potassium cyanide is added to a preparation of cytochrome c which is completely void of superoxide dismutase, there will be no effect on its rate of reduction. Conversely, if potassium cyanide is added to a preparation of cytochrome c containing trace amounts of superoxide dismutase then the rate of reduction will increase. The difference in the rate of reduction coincides with the amount of superoxide dismutase present.

Nitroblue Tetrazolium

Nitroblue tetrazolium is a highly soluble, yellow compound that forms a blue product upon reduction. This blue product known as formazan is insoluble and precipitates quickly. Nitroblue tetrazolium is well suited for the gel stain in electrophoresis, however in spectrophotometric assay, its use may be limited. Addition of 0.33 mg of gelatin per mL of assay medium prevents the precipitation of formazan making its use in assays more acceptable(55,57). Like cytochrome c, nitroblue tetrazolium may be reduced by some low molecular weight compounds found in tissue homogenates.

1.6. Purpose of Present Study

The human body hosts a constant production of oxygen-derived free radicals. Although they aid in removing ingested micro-organisms, they are also involved in many disease processes. If superoxide dismutase is to be available to combat these oxygen-derived free radicals involved in disease states, it must be formulated in a stable and commercially acceptable dosage form. Since superoxide dismutase is an enzyme, it is practical that a parenteral formulation is produced.

The Winnipeg Rh Institite has done preliminary work on the production of human superoxide dismutase and has an interest in developing and evaluating a parenteral dosage form of human superoxide dismutase for therapeutic use. One of the primary objectives of the Rh Institute is to eventually conduct clinical trials using superoxide dismutase on neonates with

bronchopulmonary dysplasia as well as to evaluate superoxide dismutase's radioprotection properties during radiation therapy. In order for these studies to be implemented, a suitable dosage form is required. For a parenteral dosage form to be suitable it must be stable, sterile, free from particulate matter and pyrogens. Pharmaceutical elegance must also be kept in mind.

The present study is a joint project between the Winnipeg Rh Institute and the Faculty of Pharmacy, University of Manitoba. The main purpose of the study is to formulate human superoxide dismutase as a commericially viable and stable parenteral dosage form. The Rh Institute has conducted preliminary stability studies on superoxide dismutase in the liquid state. Their findings indicated that the solution was not sufficiently stable, and that an alternative dosage form such as lyophilization, was to be considered.

Eight formulations of superoxide dismutase were prepared using superoxide dismutase provided from the Rh Institute. The superoxide dismutase was isolated from human erythrocytes according to the Westlake *et al.* procedure(58). All formulations were prepared using aseptic technique. As a final step, the eight formulations were lyophilized. Lyophilization is a useful process which can be applied to the manufacturing of biologicals, serums, hormones and other products which may be thermolabile or unstable in solution for long periods of time. Removal of the solvent via sublimation, allows drugs or biologicals to be dried with a minimum amount of product degradation. Choice of buffers and diluents used in this study was based on already existing standard vehicles of parenterals. Four of the eight formulations contained mannitol which was added to provide a structure or

matrix to the dried residue. Table 2 lists the composition of the eight superoxide dismutase formulations used in this study.

Table 2. Superoxide Dismutase Formulations For Current Study

PRODUCT #	FORMULATION
1	SOD 7 mg/mL -in water
2	SOD 7 mg/mL -in water with 20 mg Mannitol
3	SOD 7 mg/mL -in Phosphate buffer (20 mM), pH 7.8
4	SOD 7 mg/mL -in Phosphate buffer (20 mM), pH 7.8 with 20 mg Mannitol
5	SOD 7 mg/mL -in Normal Saline
6	SOD 7 mg/mL -in Normal Saline with 20 mg Mannitol
7	SOD 7 mg/mL -in Citrate buffer (20 mM), pH 6.6
8	SOD 7 mg/mL -in Citrate buffer (20 mM), pH 6.6 with 20 mg Mannitol

Long term stability studies were conducted on all eight freeze dried formulations stored at 4°C. The study period was 1 year in length. Short term accelerated stability studies were also carried out at 57 °C and 37 °C on four of the eight formulations following reconstitution. Likewise stability studies were also performed on four of the eight formulations at 4 °C and room temperature after reconstitution. It was anticipated that the study would illustrate suitable stability of at least one formulation to allow the possibility of further animal and clinical studies as well as eventual production of a commericially viable product.

Along with the stability studies, the enzyme was tested at various stages of the study for possible biochemical alterations of the protein. SDS-polyacrylamide gel electrophoresis was performed routinely throughout the one year period and was used to establish the molecular weight of superoxide dismutase as well as to monitor any changes that may have occured over the study period. Isoelectric focusing was another technique used to determine the pI value of the enzyme. Lastly, fast protein liquid chromatography was used to detect any changes or differences between the formulations.

CHAPTER II

EXPERIMENTAL

2.1. Chemicals and Equipment

2.1.1. Chemicals

1. Bovine Superoxide Dismutase: Lot B-7. Obtained from Dr. A. Petkau's laboratory at the Atomic Energy of Canada, Whiteshell, Manitoba.

2. Riboflavin: Lot 16F-0216 Sigma, St. Louis, Mo., USA.

3. DL-Methionine: Lot 126f-0307 Sigma, St. Louis, Mo., USA.

4. Nitroblue Tetrazolium: Lot 95f-5019 Sigma, St. Louis, Mo., USA.

5. Potassium Phosphate Monobasic; Lot 781355 Fisher Scientific Company, Fair Lawn, New Jersey, USA.

6. Potassium Phosphate Dibasic: Lot 723651 Fisher Scientific Company, Fair Lawn, New Jersey, USA.

7. Sodium Hydroxide: Lot 733478 Fisher Scientific Company, Fair Lawn, New Jersey, USA.

8. Tris(hydroxymethyl)aminomethane: Control # M1352 Bio-Rad, Richmond, California, USA.

9. N,N'-Methylene-bis-acrylamide; Control # 34179 Bio-Rad, Richmond, California, USA.

10. Acrylamide > 99.9%: Control # 28900 Bio-Rad, Richmond, California, USA.

11. Sodium Dodecyl Sulfate: Control # m1451 Bio-Rad, Richmond, California, USA.

12. Glycerol: Lot 855344 Fisher Scientific Company, Fair Lawn, New Jersey, USA.

13. Ammonium Persulfate: Control # m1134 Bio-Rad, Richmond, California, USA.

14. Temed: Control # 27946 Bio-Rad, Richmond, California, USA.

15. Brilliant Blue R (Coomassie Brilliant Blue R): Lot 87F-5001 Sigma, St. Louis, Mo., USA.

16. 2-Mercaptoethanol: Lot 29C-1924 Sigma, St, Louis, Mo., USA.

17. Dimethyldichlorosilane solution 2%(w/v) in 1,1,1-Trichloroethane (Repel-Silane): Batch # 7655430E LKB, Bromma, Sweden.

18. Trichloroacetic Acid: Lot 850330 Fisher Scientific Company, Fair Lawn, New Jersey, USA.

19. Sulphosalicylic Acid: Lot 319281 J.T. Baker Chemical Company, Phillipsburg, New Jersey, USA.

20. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Molecular Weight Standards-LOW: Control # 32569 Bio-Rad, Richmond, California, USA.

21. Isoelectric Focusing Standards(IEF) Broad Range pI 4.6-9.6: Control # 29001 Bio-Rad, Richmond, Calfornia, USA.

2.1.2. Solvents

1. Methanol: Lot CL807158 Fisher Scientific Company, Nepean, Ontario, Canada.

2. Acetic Acid: Fisher Scientific Company, Nepean, Ontario, Canada.

3. Ethanol: Fisher Scientific Company, Nepean, Ontario, Canada.

2.1.3. Stock Solutions

1. Monomer Solution (30%T 2.7%C)

Acrylamide		58.4 g
N,N'-Methylene-bis-acrylamide		1.6 g
Water	qs ad.	200.0 mL

2. 4X Running Gel Buffer (1.5M Tris-Cl pH 8.8)

Tris(hydroxymethyl)aminomethane	36.3 g
(adjust pH to 8.8 with HCl)	
Water	200.0 mL

3. 4X Stacking Gel Buffer (0.5M Tris-Cl pH 6.8)

Tris(hydroxymethyl)aminomethane	3.0 g
(adjust pH to 6.8 with HCl)	
Water	200.0 mL

4. 10% Sodium Dodecyl Sulfate

Sodium Dodecyl Sulfate		50.0 g
Water	qs ad	500.0 mL

5. Initiator (10% Ammonium Persulfate)

Ammonium Persulfate		0.5 g
Water	qs ad	5.0 mL

6. Running Gel Overlay (0.375M Tris-Cl pH 8.8, 0.1% SDS)

Stock Solution #2		25.0 mL
Stock Solution #4		1.0 mL
Water	qs ad	100.0 mL

7. 2X Treatment Buffer (0.125M Tris-Cl pH 6.8, 4% SDS, 20% Glycerol,10% 2-Mercaptoethanol)

Stock Solution #3		25.0 mL
Stock Solution #4		4.0 mL
Glycerol		2.0 mL
2-Mercaptoethanol		1.0 mL
Water	qs ad	100.0 mL

8. SDS-PAGE 5% Gel

30%T 2.7%C	3.3 mL
Stock Solution #2	5.0 mL
10% SDS	0.2 mL
Water	11.3 mL
Glycerol	0.1 mL
Ammonium Persulfate	0.1 mL
Temed	6.5 µL

9. SDS-PAGE 20% Gel

30%T 2.7%C		13.3 mL
Stock Solution #3		5.0 mL
10% SDS		0.2 mL
Water		0.5 mL
Glycerol		0.9 mL
Ammonmium Persulfate		0.1 mL
Temed		6.5 µL

10. SDS-PAGE Stacking Gel

30%T 2.7%C		2.7 mL
Stock Solution #3		5.0 mL
10% SDS		0.2 mL
Water		12.2 mL
Ammonium Persulfate		0.1 mL
Temed		10.0 µL

11. Electrophoresis Tank Buffer (0.025M Tris-HCl pH 8.3, 0.192M Glycine, 0.1% SDS)

Tris(hydroxymethyl)aminomethane		12.0 g
Glycine		57.6 g
Stock Solution #4		40.0 mL
Water	qs ad	4.0 L

12. Electrophoresis Stain Stock(1%Coomassie Blue R)

Coomassie Blue R		2.0 g
Water	qs ad	200.0 mL

(Stir and filter)

13. Electrophoresis Stain(0.125% Coomassie Blue R, 50% Methanol, 10% Acetic Acid)

Stock Solution #9		62.5 g
Methanol		250.0 mL
Acetic Acid		50.0 mL
Water	qs ad	500.0 mL

14. Electrophoresis Destaining Solution I

Methanol		500.0 mL
Acetic Acid		100.0 mL
Water	qs ad	1.0 L

15. Electrophoretic Destaining Solution II

Acetic Acid		700.0 mL
Methanol		500.0 mL
Water	qs ad	10.0 L

16. IEF Gel

Acrylamide 30% (29:1 Acryl:Bis)	4.3 mL
Sucrose 18.6% Solution	14.0 mL
Ampholine pH 4-6	1.0 mL
Ammonium Persulfate 1%	1.0 mL

17. IEF Fixing Solution

Trichloroacetic Acid		34.5 mL
Sulphosalicylic Acid		10.4 g
Water	qs ad	300.0 mL

18. IEF Staining Solution

Coomassie Blue R		0.35 g
Ethanol		75.0 mL
Acetic Acid		24.0 mL
Water	qs ad	300.0 mL

19. IEF Destaining Solution

Ethanol		500.0 mL
Acetic Acid		160.0 mL
Water	qs ad	2.0 L

20. Potassium Phosphate Buffer 0.5M

Potassium Phosphate Monobasic		34.5 g
Potassium Phosphate Dibasic		35.5 g
Water	qs ad	1.0 L

Adjust pH to 6.8

21. Running Buffer for FPLC

Sodium Chloride		17.5 g
Potassium Chloride		100.0 mL
Water	qs ad	1.0 L

Adjust pH to 6.8

2.1.4. Supplies

1. Syringes (1cc): Becton Dickinson and Company, Canada.

2. Needles (20 gauge): Becton Dickinson and Company, Canada.

3. Thermometer: Fisher Scientific Company, Fair Lawn, New Jersey, USA.

4. Disposable Test Tubes (13 X 100 mm): Fisher Scientific Company, Fair Lawn, New Jersey, USA.

5. Water for Injection (USP): Baxter Laboratories of Canada Limited. Malton, Ontario.

6. Alcohol Swabs: Triad Medical Products, H&P Industries, Inc. Franklin, Wi., USA.

7. Electrode Pads and Strips: Bio-Rad,Richmond, California, USA.

2.1.5. Equipment

1. pH Meter (Fisher Accumet pH meter, Model 825-m17): Fisher, Scientific Company, Fair Lawn, New Jersey, USA.

2. Balance (Mettler AE 160): Fisher Scientific Company, Fair Lawn, New Jersey, USA.

3. Spectrophotometer (Ultrospec II): Model 4050, LKB Biochrom, Science Park, Cambridge, England

4. Light Chamber: The light chamber consisted of 2 lamps. -Model M-208 Dazor 118 volts. Dazor Mfg. Corp. St. Louis, Mo., USA. with 3 cool white 20 cm tubes, General Electric, Canada.

5. Isoelectric Focusing apparatus (LKB 2117 Multiphor II Electrophoresis Unit): LKB Biochrom, Science Park, Cambridge, England.

6. Power Supply (LKB 2297 Macrodrive 5 Constant Power Supply,): LKB Biochrom, Science Park, Cambridge, England.

7. Water Bath (Haake G. Water Bath,): West Germany.

8. Stir Plate (Sybron/Thermolyne Nuova II Stir Plate): Model # S18525, Thermolyne Corporation, Iowa, USA.

9. Electrophoresis Tank (BIO-RAD Protean II Tank): Bio-Rad, Richmond, California, USA.

10. Fast Protein Liquid Chromatograghy(FPLC) Components: Pharmacia, Laboratory Separation Division, Uppsala, Sweden.
    -Superose 12 gel permeation column with pre filter
    -Pump P500
    -Gradient programmer, GP-250
    -Automatic injector, ACT-100, complete with motorized V7 valve and peristaltic pump PL, manual valve V7, and switching valve V8
    -Single path UV-1 optical unit, single path monitor UV-1 control unit, 280 nm filter and high resolution flow cell.

2.2. Methodology

2.2.1. Standardization and Calibration of The Superoxide Dismutase Assay

The method used was based on a photoreduction reaction of nitroblue tetrazolium according to Beauchamp and Fridovitch (49), with some minor modifications.

This assay procedure was standardized using a sample of bovine superoxide dismutase with known activity. The sample was obtained from Dr. A. Petkau's laboratory of the Atomic Energy of Canada, Whiteshell, Manitoba. The reference bovine superoxide dismutase sample had an activity of 3200 u/mg. A solution of the bovine superoxide dismutase was prepared resulting in a concentration of 30 μg/mL. Six disposible test tubes were

labeled accordingly. Test tubes 2 to 5 contained 360 ng, 720 ng, 1080 ng, and 1440 ng of bovine superoxide dismutase respectively. Test tubes 1 and 6 did not contain any bovine superoxide dismutase and were used as controls. To each test tube, 3 mL of reaction mixture was added such that each test tube contained 1.17 X 10^{-6} M riboflavin, 0.01 M methionine, 5.6 X 10^{-5} M nitroblue tetrazolium, and 0.05 M potassium phosphate at a pH 7.8. The temperature was kept constant at 21 ˚C. The addition of the reaction mixture was done under darkened conditions. The test tubes were then placed into the light chamber which resulted in an increased absorbance at 560 nm, which was linear with respect to time of illumination. This procedure was carried out several times under the same conditions changing only the time of illumination until the result of the assay corresponded with the known activity of the standard bovine superoxide dismutase. When this was established and easily reproducible, the time of illumination was then used as the standard time of illumination for assaying the freeze dried formulations of superoxide dismutase.

2.2.2. Assay For Superoxide Dismutase in Formulation

The method used was the same as for the standardization and calibration of the superoxide dismutase assay according to Beauchamp and Fridovitch with some minor modifications(49). One vial from each formulation was reconstituted with 1.0 mL sterile Water for Injection USP. This resulted in a concentration of 7 mg/ml of superoxide dismutase. A sample (42.9 µL) from each vial was placed into separate 10 ml volumetric flasks and brought up to volume with distilled water giving a final concentration of 30 µg/mL of superoxide dismutase. Six disposible test tubes were labeled accordingly.

Test tubes 2 to 5 contained 360 ng, 720 ng, 1080 ng, and 1440 ng of superoxide dismutase respectively. Test tubes 1 and 6 did not contain any superoxide dismutase and were used as controls. To each test tube, 3 mL of a reaction mixture was added such that each test tube contained 1.17×10^{-6} M riboflavin, 0.01 M methionine, 5.6×10^{-5} M nitroblue tetrazolium, and 0.05 M potassium phosphate at pH 7.8. The temperature was kept constant at 21 °C. The addition of the reaction mixture was done under darkened conditions. The test tubes were then placed in the light chamber for 3 1/4 minutes (time derived from the standardization and calibration of the bovine superoxide dismutase). This procedure was repeated once for each formulation of superoxide dismutase. The absorbance was read at 560 nm.

2.2.3.1. SDS-Polyacrylamide Gel Electrophoresis

The SDS polyacrylamide gel electrophoresis was carried out using a gel with a gradient from 5-20%. The gels were made in accordance to the formuation given for stock solutions 8 and 9. The 5% and 20% gels were prepared in separate flasks. All chemicals were added to the flask excluding the initiators, that is, the ammonium persulfate and temed. Degassing of the gel solutions was done using a vacuum suction to remove oxygen which may prevent the polymerization of acrylamide. Buffer stock and monomer solution were stored at 4 °C and thus have a greater capacity to dissolve oxygen. Degassing took place at room temperature for at least 5 minutes. After degassing was complete the ammonium persulfate and temed were added to the flasks. The contents of the flasks were then transfered into the gradient maker. From the gradient maker the gel solutions were poured between two glass plates until the solutions reached 4 cm from the top of the

glass plates. The glass plates were held tightly in place. Butanol was placed on top of the gel to prevent the formation of a rough surface. The gel sat for 1 hour after which the butanol was removed.

A stacking gel was prepared according to stock solution 10. As with the running gel, the initiators were omitted until after degassing was complete. Once the initiators were added, the stacking gel was placed on top of the running gel. A space of approximately 1 cm was left from the top of the glass plates in order to allow for overflow upon the placement of the well-makers. After the well-maker was in place, butanol was again added. After 1 hour of standing, the well-maker was removed and the butanol was flushed out using tank buffer.

The gels were then placed into the Protean II Bio-Rad electrophoretic apparatus. The tank was attached to the water bath which continually circulated water around the prepared gels at 20 ˚C. Sufficient tank buffer was added to ensure the gels were completely covered. Samples and markers were added to the wells and the power supply was set. Electrophoresis was carried out at 1000 V, 25 W, and 30 mA for a period of 5 1/2 hours.

After electrophoresis was completed, the power pack was turned off and the apparatus was dismantled, and the gels were removed carefully. The gels were placed into a vessel containing the electrophoretic staining solution. The gels remained in the staining solution overnight. On the following day the gels were removed from the staining solution and were placed in the electrophoretic destaining solution I. After soaking in the destaining solution

I for 1 hour, the gels were carefully transfered to the electrophoretic destaining solution II and left for 1 day.

2.2.3.2. Preparation of Standards and Samples For SDS-PAGE

Superoxide dismutase samples, (250 μg) were taken from each of the eight formulations and made up to a final volume of 250 μL. To each of these samples, 250 μL of 2X treatment buffer with 2-mercaptoethanol was added. The samples were then placed into a hot water bath and allowed to boil for 5 minutes. After removing from the heat, the samples were placed on ice to cool down before being applied to the wells. The SDS-PAGE molecular weight standard used was prepared in an identical manner. 30 μL aliquots, of both standard and sample were then placed into separate wells using a micropipette.

2.2.4.1. Isoelectric Focusing(IEF)

A 0.5 mm gel was prepared using the Ultramould-gel casting kit by Bio-Rad. Glass plates were washed thoroughly with detergent and distilled water, and wiped clean using a lint free cloth. The short plate was coated with Repel Silane and allowed to dry. Upon drying, the plate was rinsed with distilled water and again allowed to dry. The long plate was then place on the casting frame and the frame was leveled. Light sensitive gel bond, hydrophilic side up, was rolled onto the glass plate with a thin film of water between the two. A gel spacer was then applied over top and 4 clamps and a screw were used to hold it in place. The short plate was placed back on the gel spacer with the Repel Silane side down. The gel was prepared according to the formulation

of stock solution 16. The ammonium persulfate was added to the gel mixture after degassing for 10 minutes. The gel mixture was then poured slowly with an even front while pushing the short plate across the gel band. A weight was placed on top until the gel had solidified (approximately 1 hour). The glass plates were then separated with a spatula. The plate should release with the gel left adhering to the gel bond.

The gel was placed on a precooled plate which was connected to the water bath and set at 10 °C. Electrode strips were placed on the anode and cathode. The electrode strip on the anode was soaked with glutamic acid 0.1 M in 0.5 M H_3PO_4. Likewise, the electrode strip on the cathode was soaked with L-alanine 0.1 M. The glass plate of the isoelectric focusing apparatus was place on top and the gel was prefocused for 30 minutes to remove the ammonium persulfate. The power supply was set at 1000 V and 5 W. The sample pads were then placed on the gel near the cathode. Each sample was pipetted onto the pads. The cover was again replaced and electrophoresis was carried out at 1000-2000 V, unlimited current, and 5 W(constant) for approximately 2 hours.

2.2.4.2. Preparation of Samples and Standards For Isoelectric Focusing

An aliquot comprising 100 µg of superoxide dismutase was taken from each of the eight samples and was made up to 100 µL. Samples containing 15 µL of each of these solutions were then placed onto the applicator pads. Undiluted IEF broad range standards, 15 µL, were also placed onto sample pads and used as markers.

2.2.5. Fast Protein Liquid Chromatography

Fast protein liquid chromatography (FPLC),was conducted by Pat Emerson of the Winnipeg Rh Institute. The procedure was carried out in accordance to the Rh Institute's standard operations and procedures manual. The samples were analyzed as a full strength solution. The flow rate was set at 0.33 mL/minute. The column used contained Superose-12 permeation gel with a pre filter. Solvents and buffers used are as detailed in the standard operation and procedure manual.

Three batches of superoxide dismutase were analyzed by FPLC. The first batch analyzed were products 1 through 8 which were freezed dried and stored for 12 months and then reconstituted with 1.0 mL sterile Water for Injection USP. The second FPLC run was conducted on products 1 through 8 which were reconstituted with sterile water and kept at 4 ˚C for 9 months. Lastly, products 1 through 8 which were reconstituted with sterile water and then frozen for six months were also analyzed by FPLC.

2.3. Data Analysis

2.3.1. Determination of Superoxide Dismutase Activity

As defined by McCord and Fridovitch(47), a unit of superoxide dismutase (SOD), activity is the amount of superoxide dismutase required to inhibit the rate of reduction of the detector in the assay system by 50%. In the present study the detector used was nitroblue tetrazolium. Figure 5 represents an

example of the effects of superoxide dismutase on this system. Reduction of nitroblue tetrazolium during the required 3 1/4 minutes of illumination was measured in terms of increased absorbancy at 560 nm and percent inhibition caused by superoxide dismutase was plotted as a function of the concentration of the enzyme(49). Activity can thus be calculated according to equation 1.

Activity= 1000/ Concentration of SOD at 50% Inhibition(per mL)

Equation 1. Calculation of SOD Activity

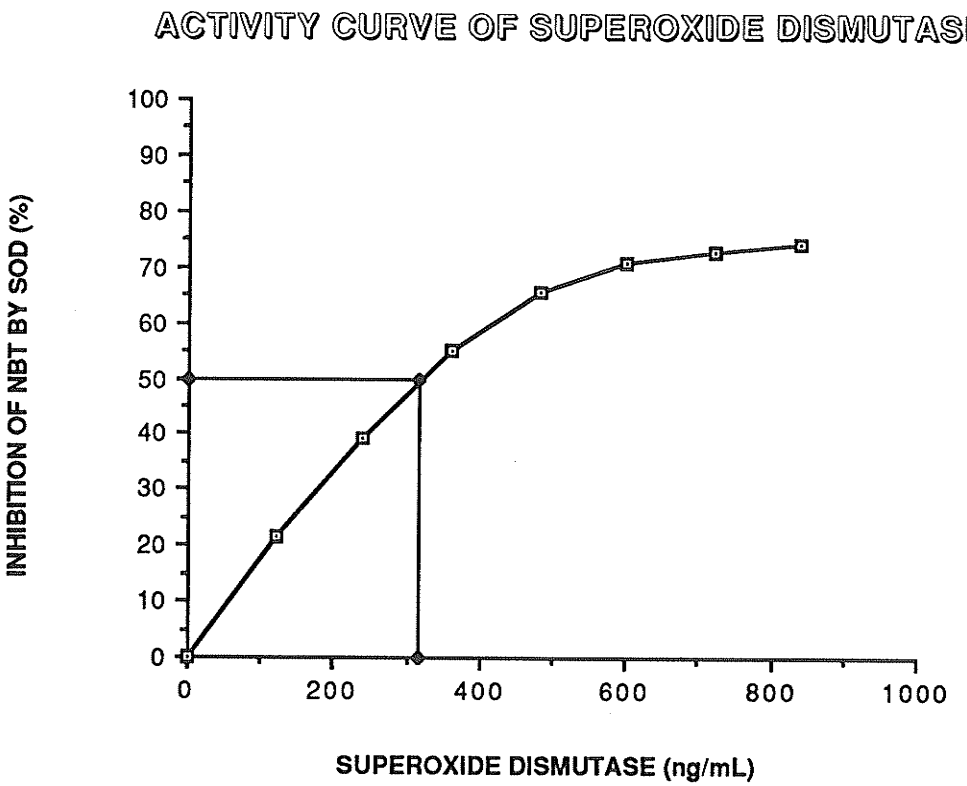


Figure 5

2.3.2. Determination of Molecular Weight Using SDS-PAGE

Estimation of protein molecular weight was determined by a routine SDS-polyacrylamide gel electrophoresis method. Molecular weight is a log function of relative mobility. The lower the molecular weight the greater the relative mobility. Low molecular weight SDS-PAGE standards ranging from 14400 to 110000 daltons were used in this study for the standard curve. The relative mobility of a protein of unknown molecular weight was fitted to the standard curve to determine its molecular weight.

A standard curve extrapolated to the y-intercept gives the molecular weight exclusion limit of that particular gel. In other words, proteins with a molecular weight of greater than the y-intercept will show zero mobility and will be excluded from the gel matrix. Fig 6 shows the standard curve obtained from the low molecular weight SDS-PAGE standards. The curve is obtained by plotting log molecular weight versus relative mobility. In this case the y-intercept vale is 5.07 as determined by linear regression analysis. Therefore the approximate exclusion limit of the gel is 118000 Daltons. The slope of the line determined by linear regression analysis was -1.14. The y-intercept value will be different for every gel percentage, however, the value should be highly reproducible from gel to gel if the same gel percentage and standards are used.

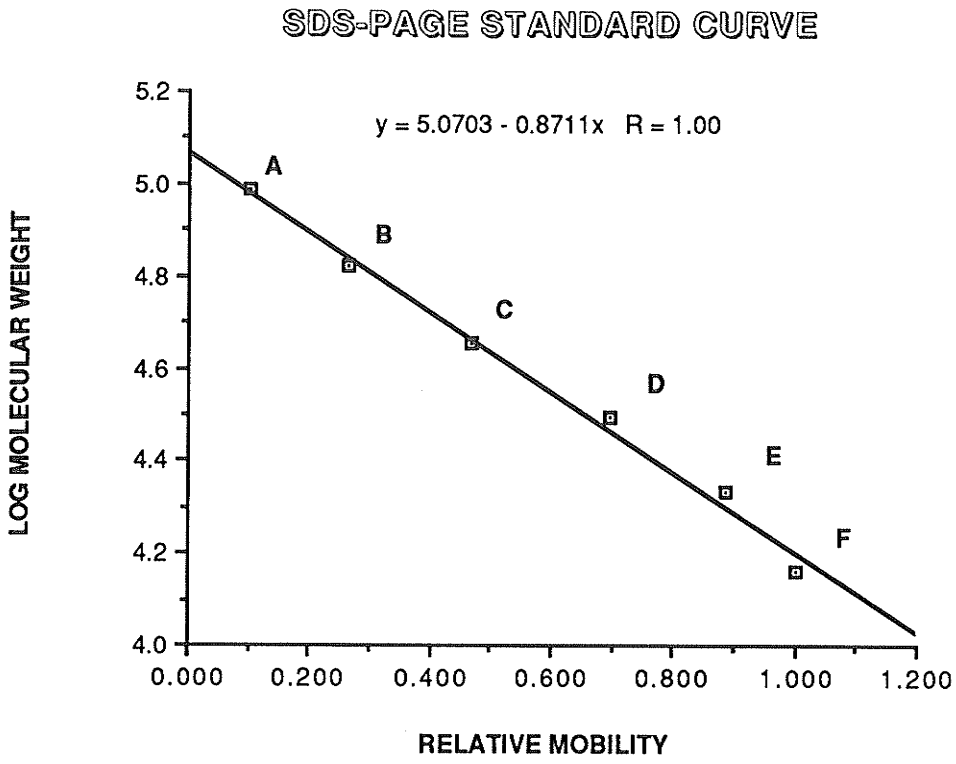


Figure 6

Table 3. Composition of SDS-PAGE Standards

SYMBOL	PROTEIN MARKER	MOLECULAR WEIGHT
A	Phosphorylase B(rabbit muscle)	97 400
B	Bovine Serum Albumin	66 200
C	Ovalbumin (hen egg white)	45 000
D	Carbonic Anhydrase (bovine)	31 000
E	Soybean Trypsin Inhibitor	21 500
F	Lysozyme (hen egg white)	14 400

2.3.3. Determination of Isoelectric Point

Once the isoelectric focusing procedure has been completed, a gradient profile calibration curve is prepared. In so doing, the distance from the cathode to each protein pI marker is measured to the nearest 0.05 cm. On a piece of graph paper, the known pI value of each pI marker protein is plotted against the distance from the cathode. The points are then connected giving the gradient profile calibration curve (fig. 7).

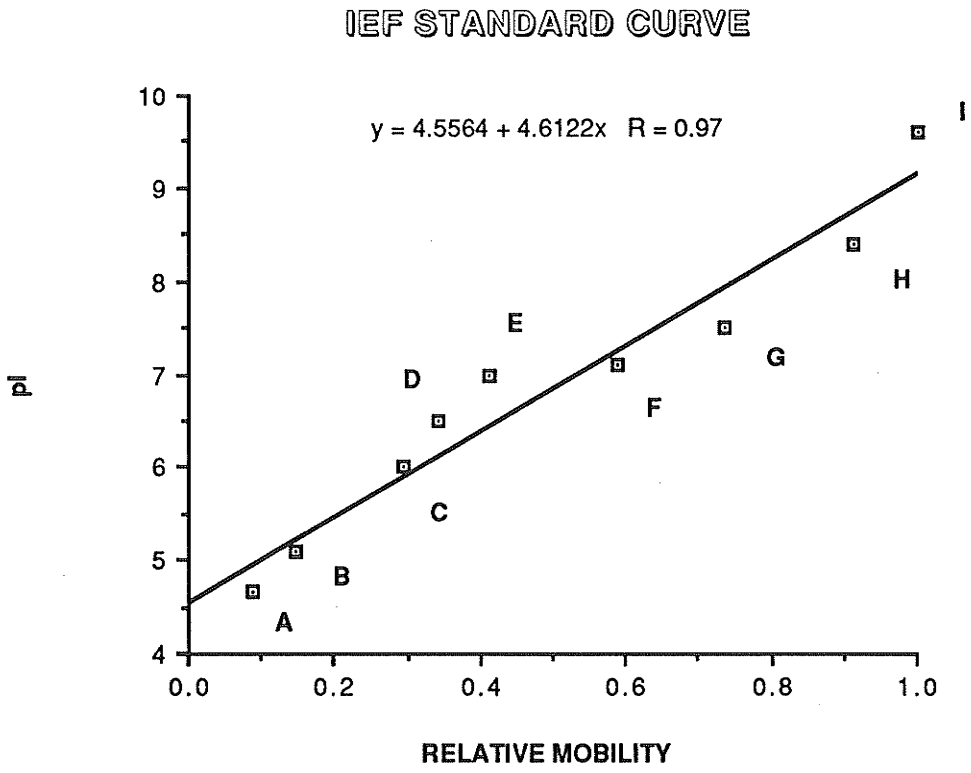


Figure 7

To determine the pI of superoxide dismutase, the distance from the cathode is measured to the nearest 0.05 cm. From the pI calibration curve prepared, the pI of superoxide dismutase can be found via extrapolation.

Table 4. Composition of Standards For IEF

SYMBOL	PROTEIN MARKER	pI
A	Phycocyanin	4.65
B	B-Lactoglobulin B	5.10
C	Bovine Carbonic Anhydrase	6.00
D	Human Carbonic Anhydrase	6.50
E	Equine Myoglobin	7.00
F	Human Hemoglobin A	7.10
G	Human Hemoglobin C	7.50
H	Lentil Lectin (three bands)	8.20, 8.40, 8.60
I	Cytochrome c	9.60

2.3.4. Fast Protein Liquid Chromatography

Fast protein liquid chromatography was a second technique used to determine molecular weight. Also, FPLC was used to monitor for any differences occuring during storage of superoxide dismutase preparations. Molecular weight is a log function of retention time. Molecules coming off the Superose-12 column will do so in order of decreasing molecular size.

Figure 8 represents an FPLC run using standards with known molecular weights. From this, a standard curve was prepared by plotting log molecular weight vs retention time (figure 9). With the standard curve prepared, molecular weight estimations were determined by plotting the time required for elution of the peaks and extrapolating from the curve the corresponding log of the molecular weight.

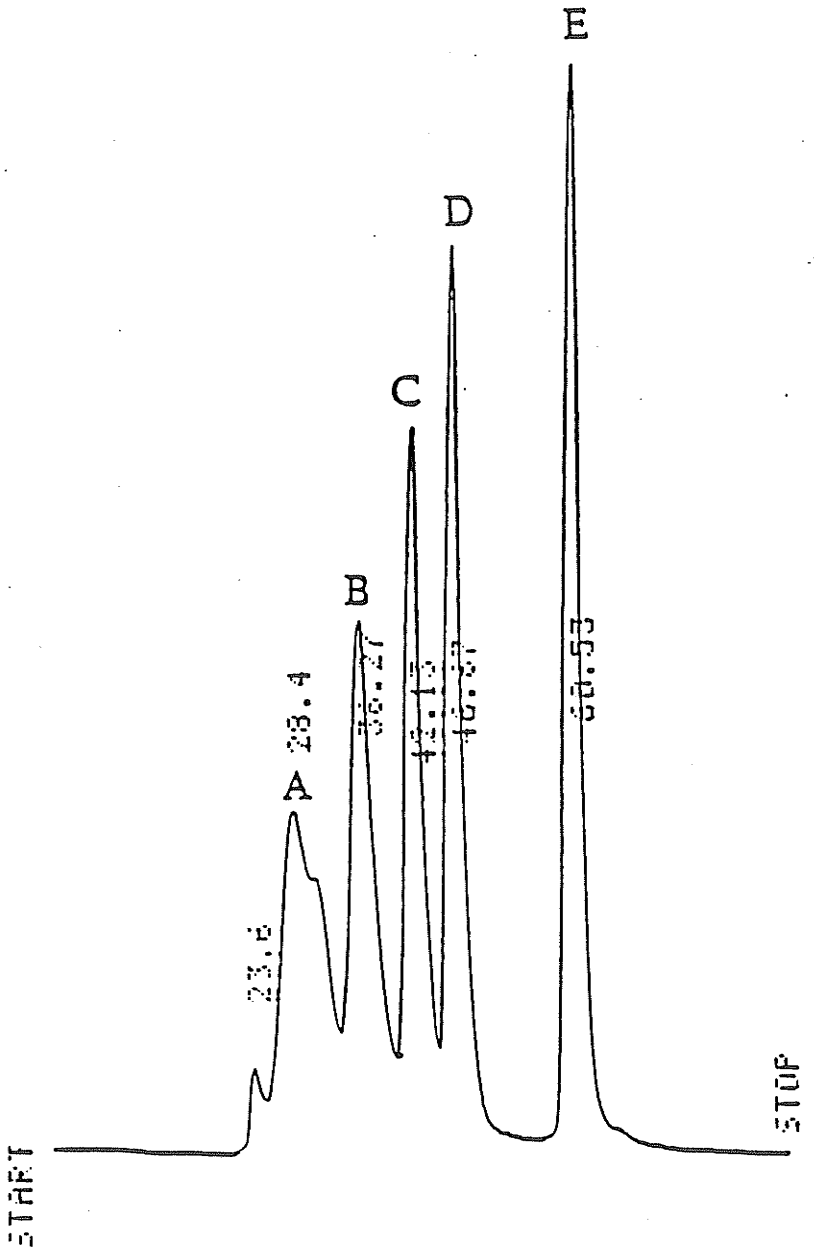


Figure 8. FPLC of Standards

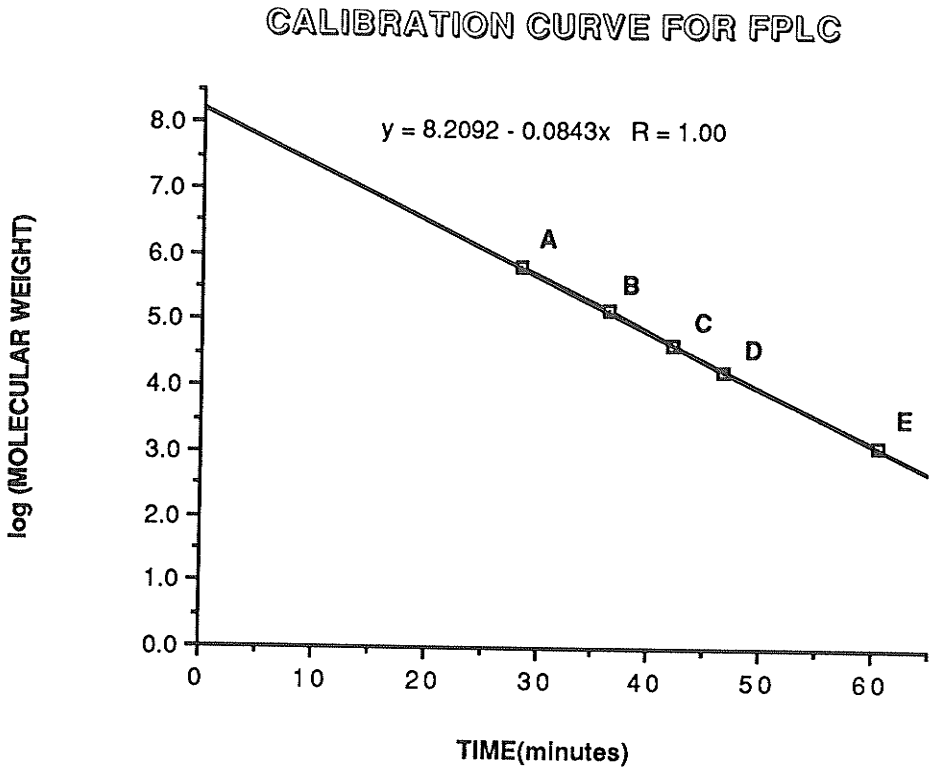


Figure 9

Table 5. Composition of FPLC Standards

SYMBOL	PROTEIN MARKER	MOLECULAR WEIGHT
A	Thyroglobin	640 000
B	Bovine Gamma Globulin	158 000
C	Ovalbumin	44 000
D	Myglobulin	17 000
E	Cyanocobalamin	1350

Aside from molecular weight determination, FPLC was also used to detect differences between samples. In this study, three batches were analyzed by FPLC. The first batch of products was freshly reconstituted from the freeze dried state. The second batch of products were kept for 9 months at 4 ˚C after being reconstituted from the freeze dried dosage form. Lastly, the third batch of products were reconstituted and then refrozen for 6 months. Each batch consisted of products 1 throught 8. The three batches were compared for number of major peaks, time of peaks, and relative area under the peaks which represents relative concentrations.

Capter III

RESULTS

3.1. Superoxide Dismutase Assays

3.1.1. Calibration and Standardization of SOD Assay

Several assays of bovine superoxide dismutase were run to determine the time of illumination required to give reproducible readings of 3200 u/mg, corresponding to the actual activity of the bovine superoxide dismutase. In the study it was found that an illumination time of 3 1/4 minutes gave consistent activity readings of approximately 3200 u/mg within an acceptable range. Table 6 lists 26 activities calculated using a illumination time of 3 1/4 minutes.

Table 6. Activity of Bovine Superoxide Dismutase at t=3 1/4 Minutes

RUN	ACTIVITY(u/mg)	RUN	ACTIVITY(u/mg)
1	3100	14	3000
2	3000	15	3400
3	3200	16	3500
4	3000	17	3300

Table 6 Cont'd			
5	3200	18	3100
6	3200	19	3100
7	3000	20	3400
8	3200	21	3400
9	3300	22	3200
10	3200	23	3000
11	3200	24	3100
12	3200	25	3400
13	3200	26	3200

Table 7. Statistical Parameters of Bovine Superoxide Dismutase Activities For The Standardization of The Assay

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE
MODEL	1	53250.427	52350.427
ERROR	24	457264.957	19052.707
CORRECTED TOTAL	25	509615.385	
MODEL F=2.75			
R-SQUARE	C.V.	ROOT MSE	Y MEAN
0.102725	4.3187	138.03154188	3196.15384615

3.1.2. Long Term Human Cu,Zn Superoxide Dismutase Stability Study on Freeze Dried Products

From the standardization of the superoxide dismutase assay it was established that the time of illumination required for a reproducible system was 3 1/4 minutes. The freeze dried products were stored at 4 °C. The formulations were reconstituted just prior to being assayed. The assay was carried out at 21 °C and each vial was assayed twice. Table #8 lists the activities of the various formulations of superoxide dismutase throughout a period of one year.

Table 8. Activity of Superoxide Dismutase(u/mg) of Freeze Dried Products

	TIME(MONTHS)							
	0	1	2	3	5	9	10	12
PROD	2300	2500	2400	2300	2500	2500	2500	2300
1	2500	2300	2500	2300	2500	2400	2300	2300
PROD	2500	2300	2400	2300	2400	2500	2300	2300
2	2400	2400	2500	2400	2300	2400	2400	2500
PROD	2400	2500	2300	2400	2500	2500	2400	2300
3	2300	2300	2500	2300	2300	2300	2500	2400
PROD	2400	2300	2300	2300	2500	2300	2500	2300
4	2500	2500	2400	2300	2300	2500	2300	2500
PROD	2500	2400	2400	2400	2400	2500	2500	2400
5	2300	2400	2400	2500	2300	2300	2400	2500
PROD	2300	2300	2400	2300	2300	2300	2400	2400
6	2500	2400	2300	2400	2300	2300	2400	2400
PROD	2500	2300	2300	2500	2500	2500	2300	2500
7	2400	2500	2400	2400	2400	2300	2400	2400
PROD	2400	2300	2500	2300	2300	2300	2300	2500
8	2500	2300	2500	2500	2400	2500	2300	2400

Table 9. Statistical Parameters of Superoxide Dismutase Activities For Long Term Stability Study

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE
MODEL	63	328671.875	5217.0139
ERROR	64	555000.000	8671.875
CORRECTED TOTAL	127	883671.875	
MODEL F=0.6			PR > F=0.9775

R-SQUARE	C.V.	ROOT MSE	Y MEAN
0.391939	3.8915	93.12290266	239296875

SOURCE	DF	TYPE I SS	F VALUE	PR > F
TIME	7	30546.875	0.05	0.8287
FORMULA	7	35546.875	0.59	0.7652
FORMULA* TIME	49	262578.125	0.62	0.9596

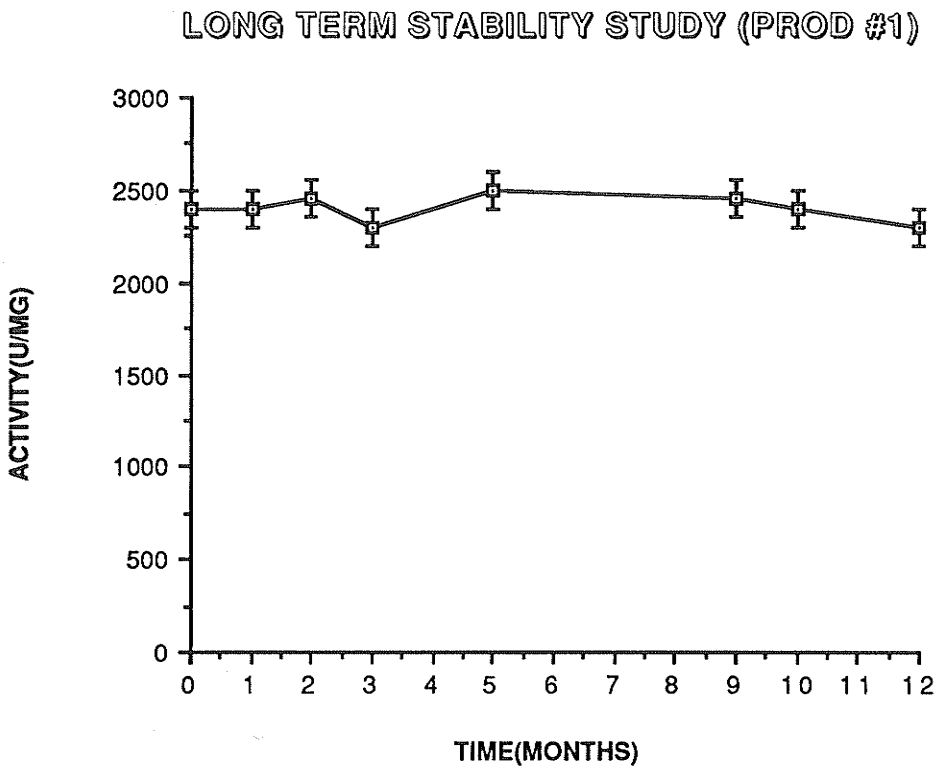


Figure 10

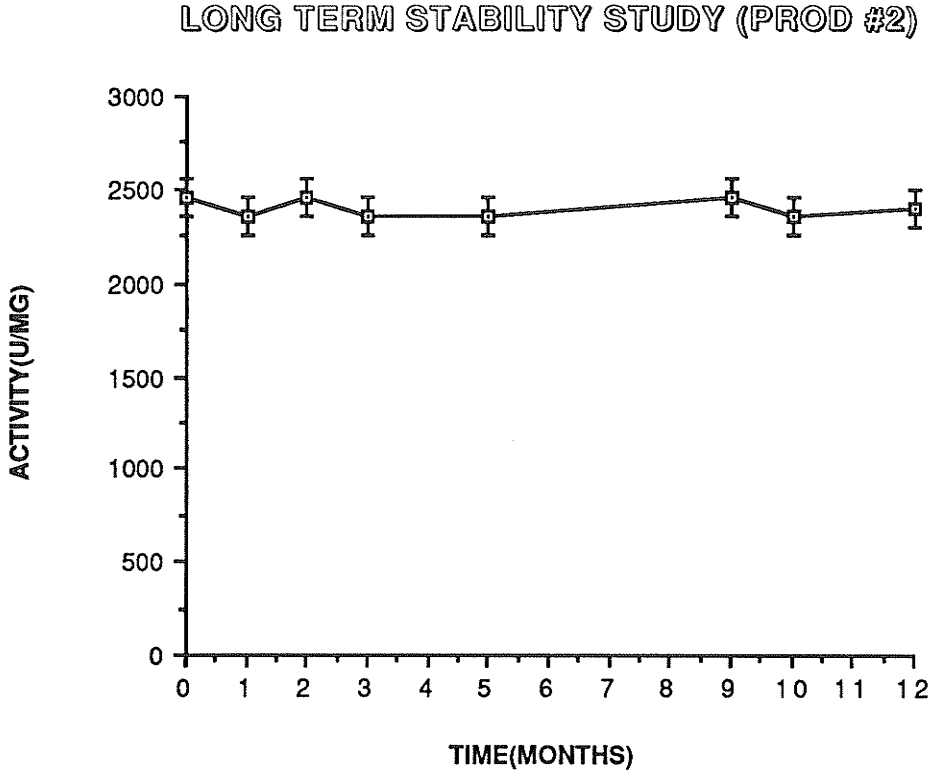


Figure 11

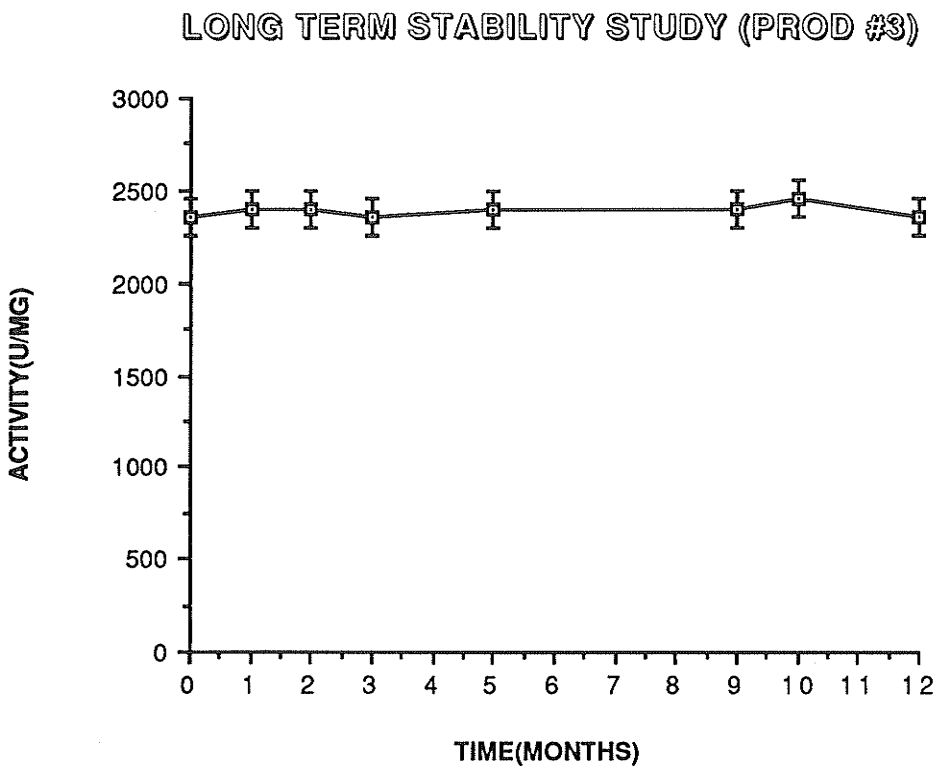


Figure 12

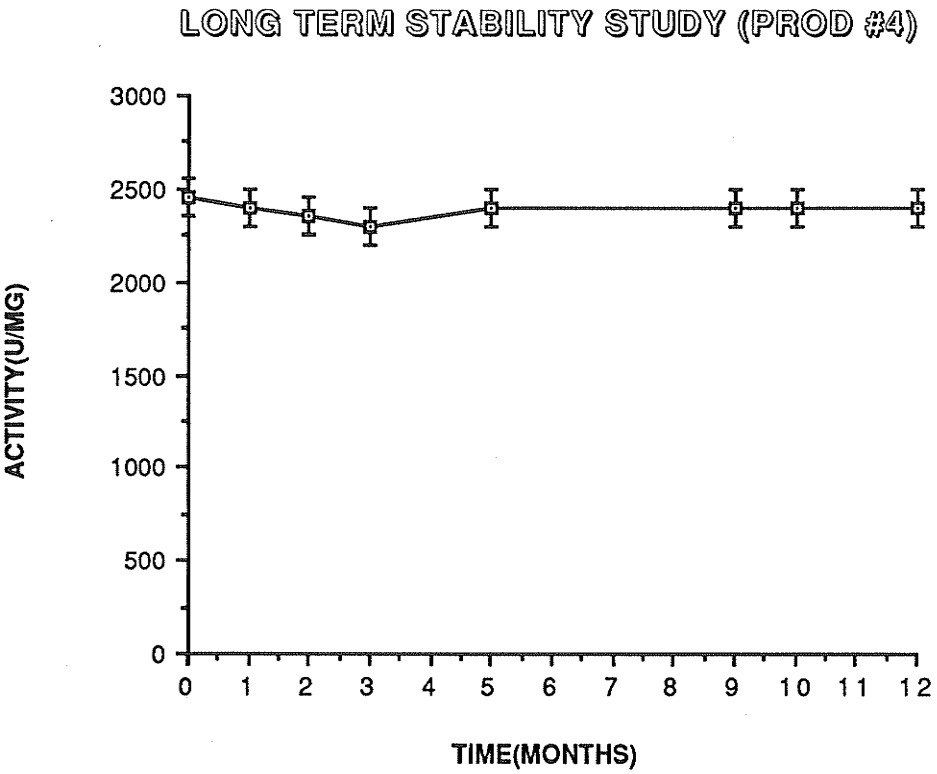


Figure 13

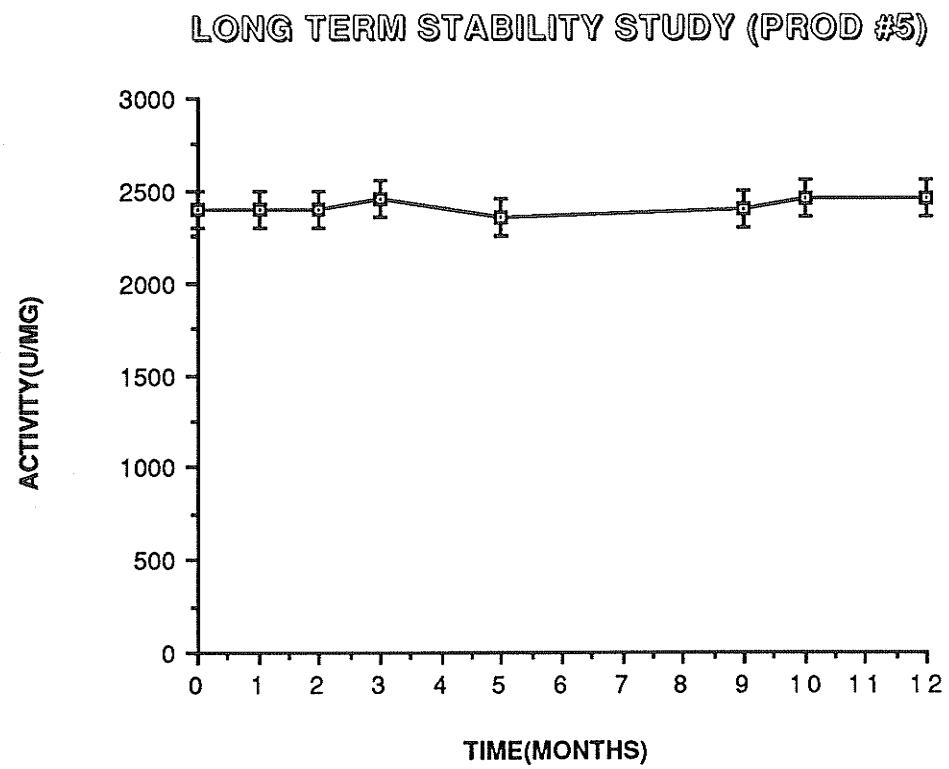


Figure 14

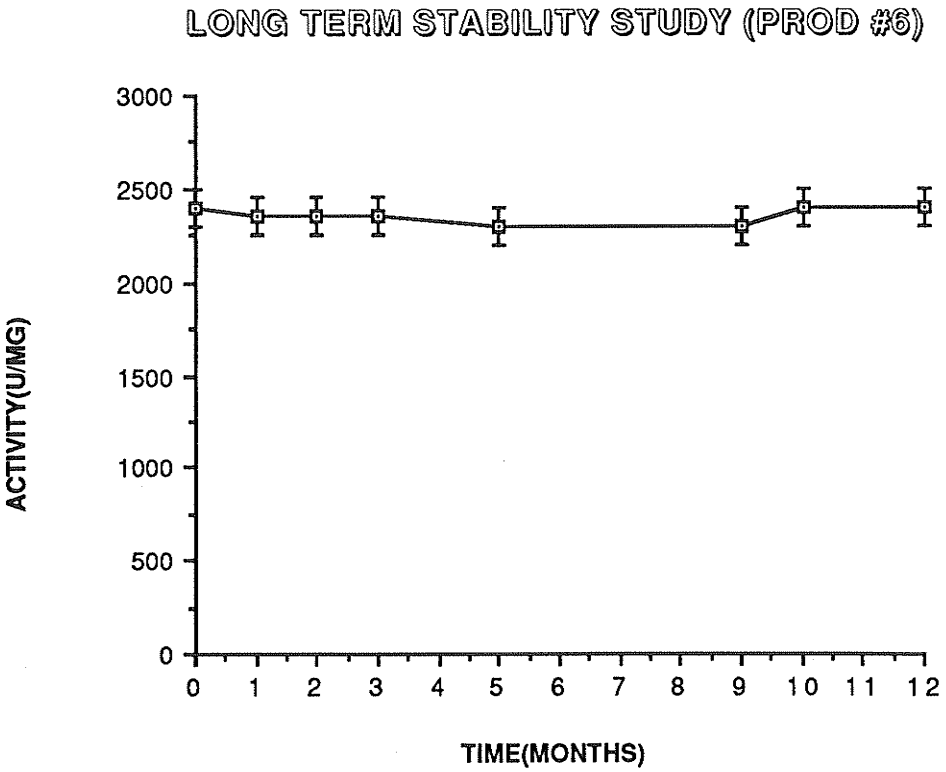


Figure 15

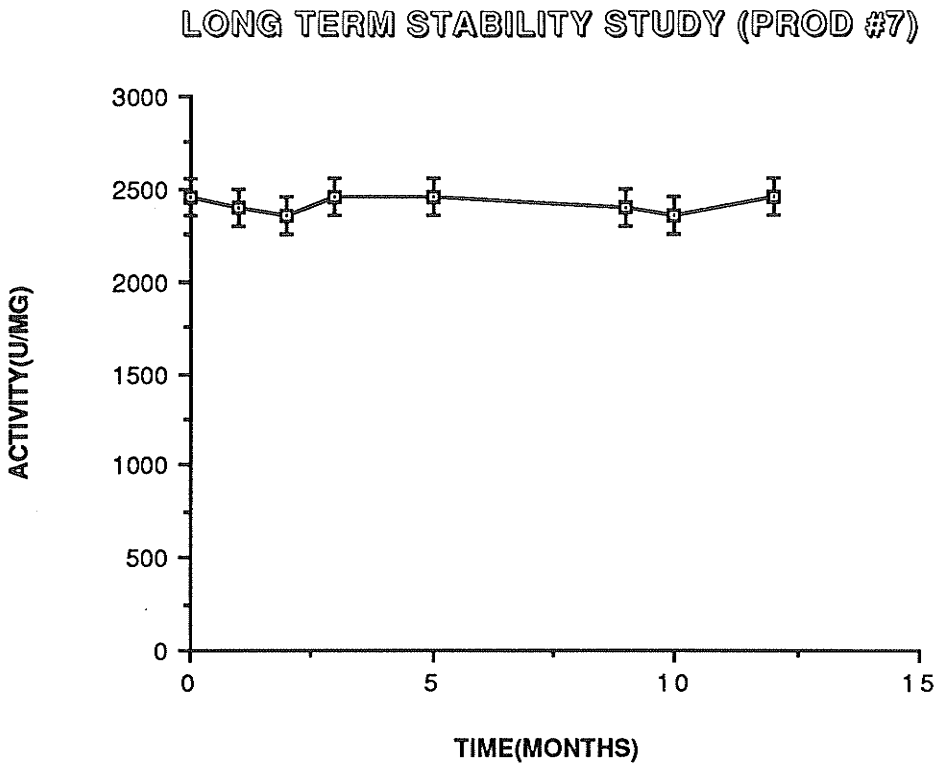


Figure 16

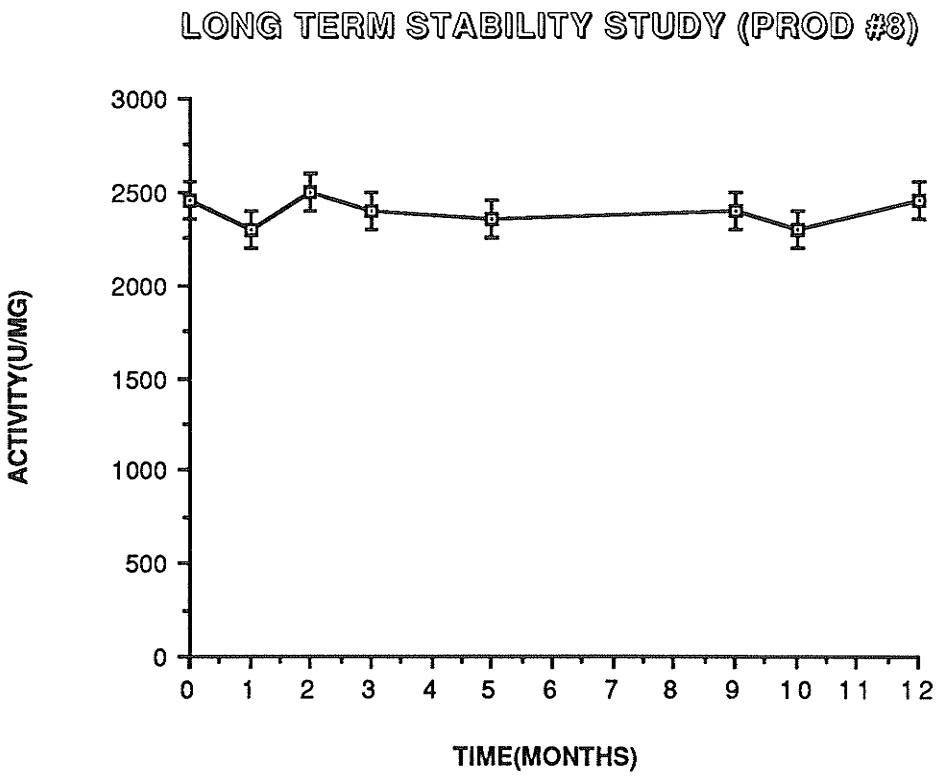


Figure 17

3.1.3. Accelerated Stability Study at 57 ˚C

In this study, the 4 formulations containing mannitol were tested for superoxide dismutase activity. Each vial was reconstituted with 1.0 mL sterile Water for Injection USP. and placed into the water bath at 57 ˚C. Table 10 lists the activities of these four preparations at 57 ˚C with an illumination time of 3 1/4 minutes.

Table 10. Activity of Superoxide Dismutase at 57 ˚C

TIME(HOURS)	PROD. 1	PROD. 3	PROD. 5	PROD. 7
0	2300	2300	2300	2300
	2300	2400	2500	2400
0.5	1900	1900	2100	2000
	2000	2000	2100	2100
1	1900	1800	1700	1700
	1900	1600	1800	1900
2	1800	1500	1700	1700
	1900	1400	1600	1800
3	1800	1200	1300	1600
	1800	1200	1400	1700
4	1600	1000	1200	1500
	1700	1000	1200	1500
6	1300	700	900	1400
	1200	700	1000	1300
8	700	300	700	900
	700	400	800	1000
12	500	<200	500	700
	400	<200	500	900
20	<200	<200	<200	300
	<200	<200	<200	200

Table 11. Statistical Parameters of Superoxide Dismutase Activities For Accelerated Stability Study at 57 °C

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE
MODEL	39	40758875.000	1045099.359
ERROR	40	185000.000	4625.000
CORRECTED TOTAL	79	40943875.000	
MODEL F=225.97			PR > F=0.0001
R-SQUARE	C.V.	ROOT MSE	Y MEAN
0.995482	5.2668	68.00735254	1291.25

SOURCE	DF	TYPE ISS	F VALUE	PR > F
TIME	9	37887624.999	910.21	0.0001
FORMULA	3	1641375.000	118.30	0.0001
FORMULA* TIME	27	1229785.000	9.85	0.0001

STUDENT-NEWMAN-KEULS' TEST

ALPHA=0.05	DF=40	MSE=4625	
# OF MEANS	2	3	4
CRITICAL RANGE	43.4663	52.3439	57.6442
SNK GROUPING	MEAN	N	PROD. #
MEANS WITH SAME LETTER ARE NOT SIGNIFICANTLY DIFFERENT			
A	1445.00	20	7
B	1385.00	20	1
C	1265.00	20	5
D	1070.00	20	3

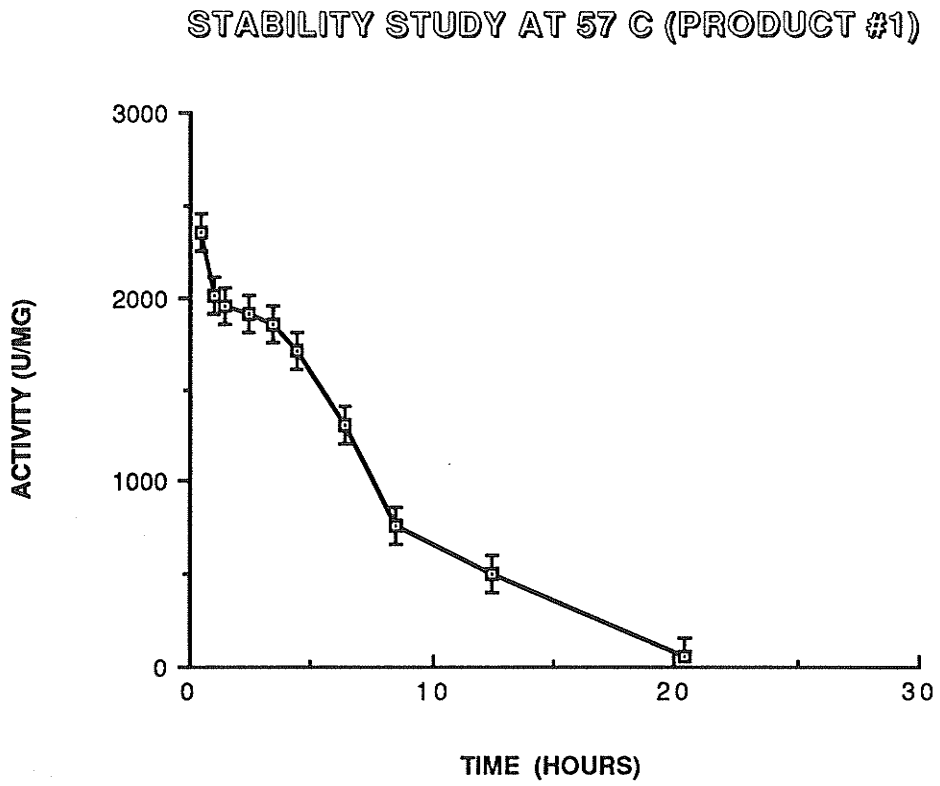


Figure 18

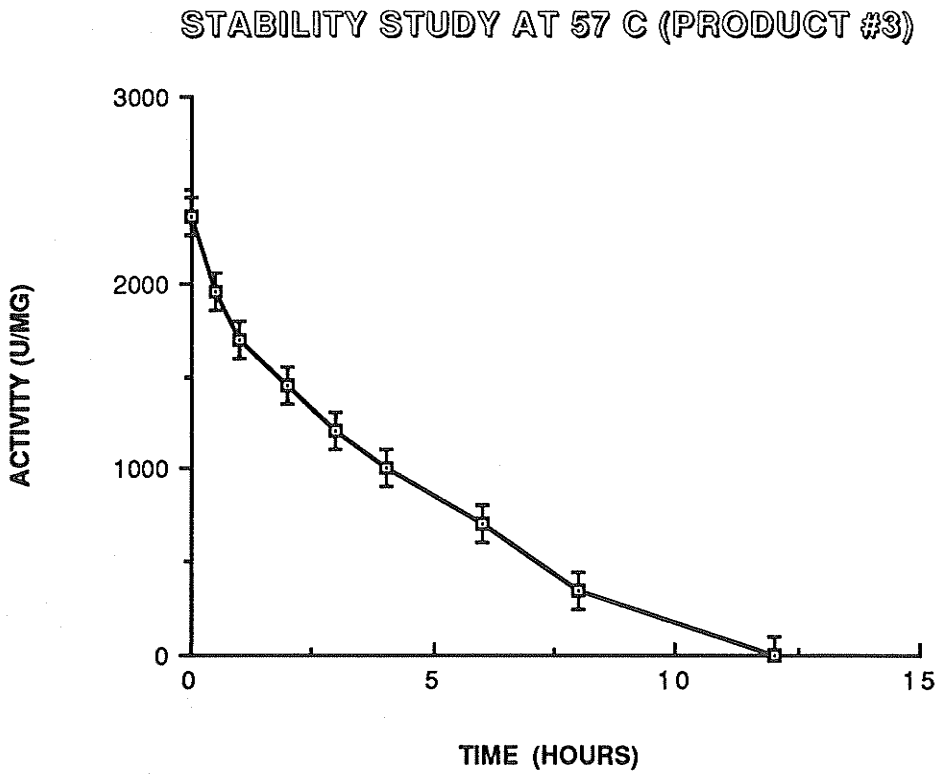


Figure 19

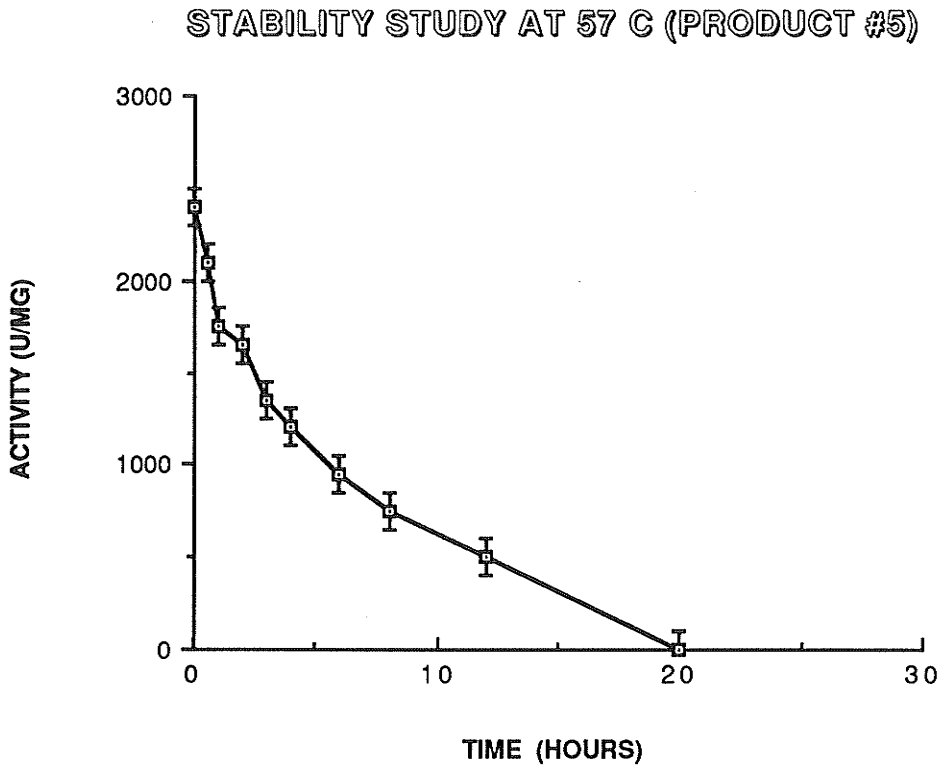


Figure 20

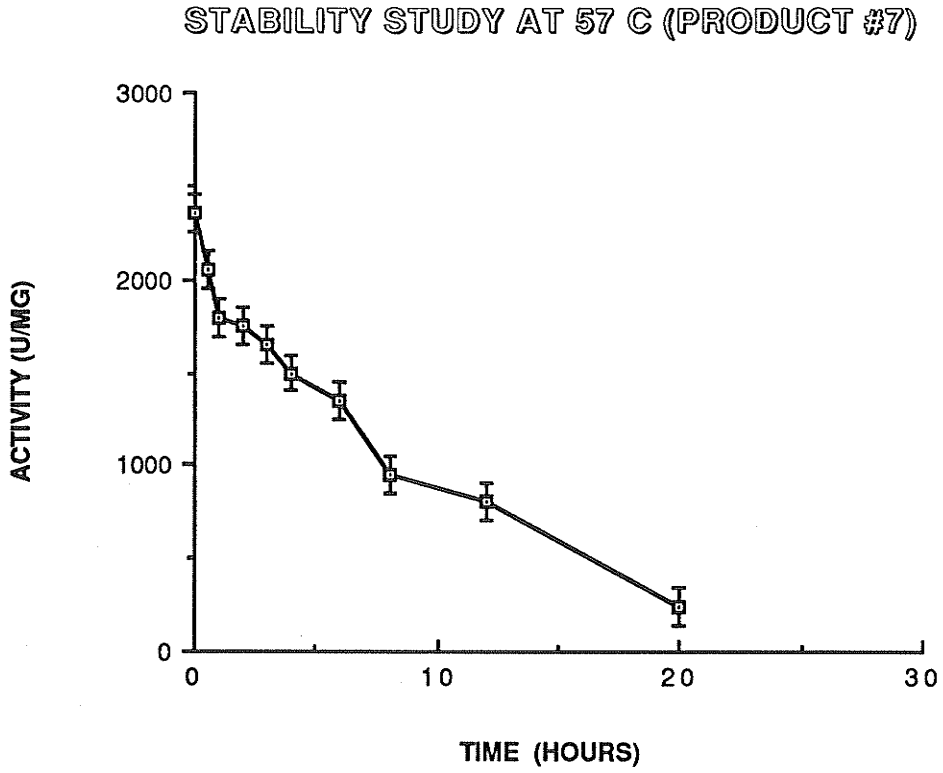


Figure 21

3.1.4. Accelerated Stability Study at 37 °C.

In this study, the four formulations containing mannitol were tested for superoxide dismutase activity. Each vial was reconstituted with 1.0 mL sterile Water for Injection USP and set in the water bath at 37 °C. Table 12 lists the superoxide dismutase activities of the four preparations with an illumination time of 3 1/4 minutes.

Table 12. Activities of Superoxide Dismutase in Formulation at 37 °C.

TIME(HOURS)	PROD. 1	PROD. 3	PROD. 5	PROD. 7
0	2400	2400	2500	2300
	2500	2500	2400	2200
1	2200	2400	2300	2300
	2100	2500	2300	2400
2	2100	2100	2100	2200
	2200	2200	2100	2300
3	2100	2000	2100	1900
	2200	1900	2000	1900
6	1900	1900	2000	1700
	1800	1800	2000	1800
12	1600	1800	1800	1800
	1700	1700	1900	1700
24	1800	1900	2000	1700
	1700	2000	1700	1600
48	1900	1900	1800	1900
	1800	2000	1800	1800
72	1800	1700	1600	1900
	1700	1800	1400	2000
168	1400	1400	1300	1700
	1500	1300	1300	1800
360	1400	1400	1300	1900
	1500	1300	1300	1900

Table 13. Statistical Parameters of Superoxide Dismutase Activities For Acclerated Stability Study at 37 ˚C

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE
MODEL	47	8195909.090	174381.044
ERROR	40	223636.363	5590.909
CORRECTED TOTAL	87	8419545.454	
MODEL F= 31.19			PR > F=0.0001
R-SQUARE	C.V.	ROOT MSE	Y MEAN
0.643438	3.9307	74.77268187	1902.272727

SOURCE	DF	TYPE I SS	F VALUE	PR > F
FORMULA	3	10681.818	0.64	0.5957
SAMPLE(FORMULA)	4	16363.636	0.73	0.5757
TIME	10	6912045.454	123.63	0.0001
TIME*FORMULA	30	1204318.181	7.18	0.0001

STUDENT-NEWMAN-KEULS' TEST

ALPHA=0.05	DF=44	MSE=5340.91	
# OF MEANS	2	3	4
CRITICAL RANGE	44.4101	53.4461	58.8322

GROUPING	MEAN	N	PROD. #
MEANS WITH SAME LETTER ARE NOT SIGNIFICANTLY DIFFERENT			
A	1940.91	22	7
B	1904.55	22	3
B	1877.27	22	1
B	1863.64	22	5

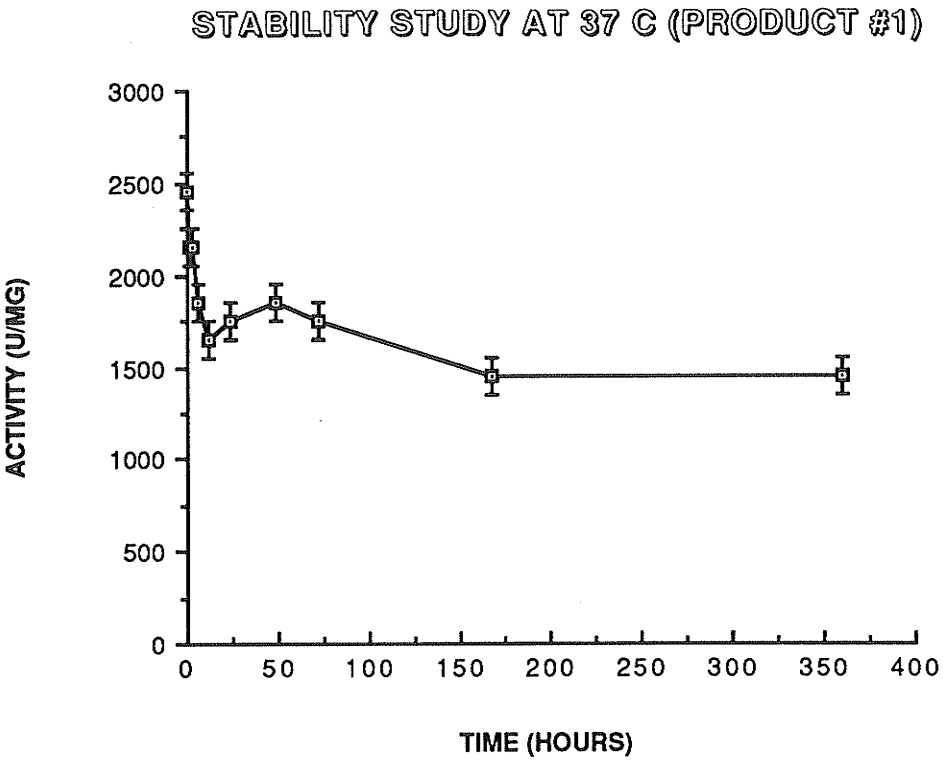


Figure 22

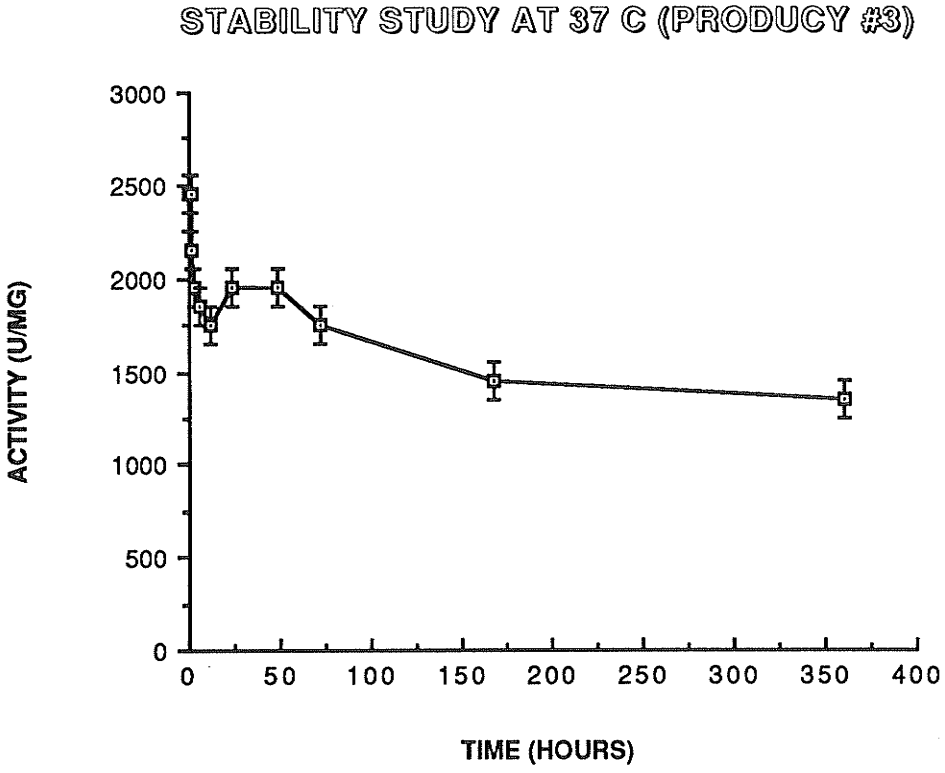


Figure 23

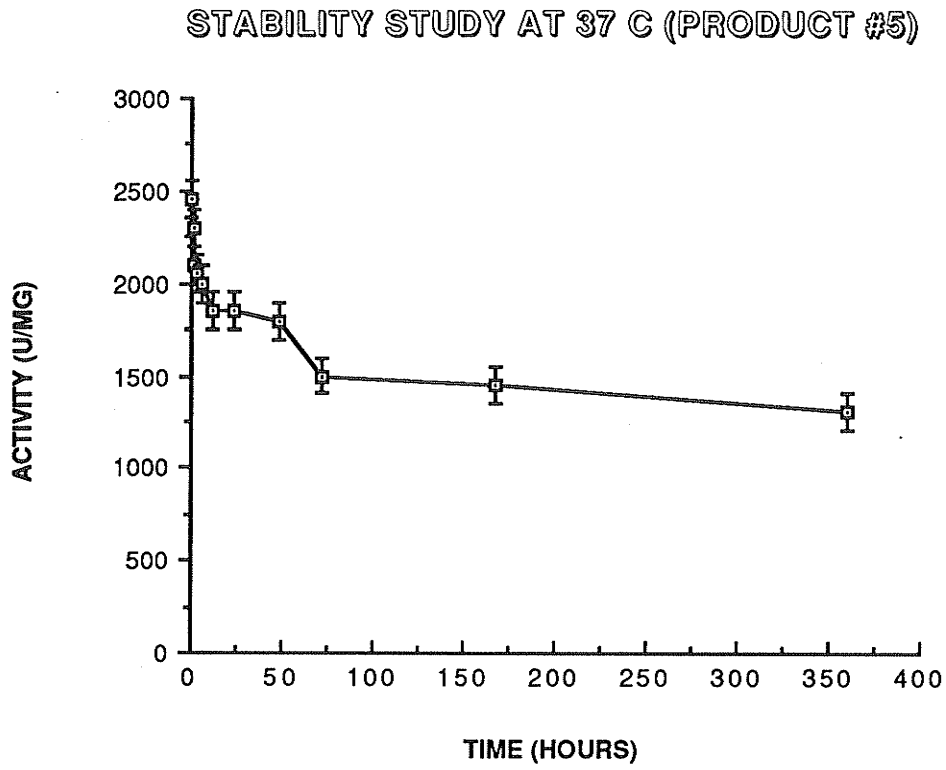


Figure 24

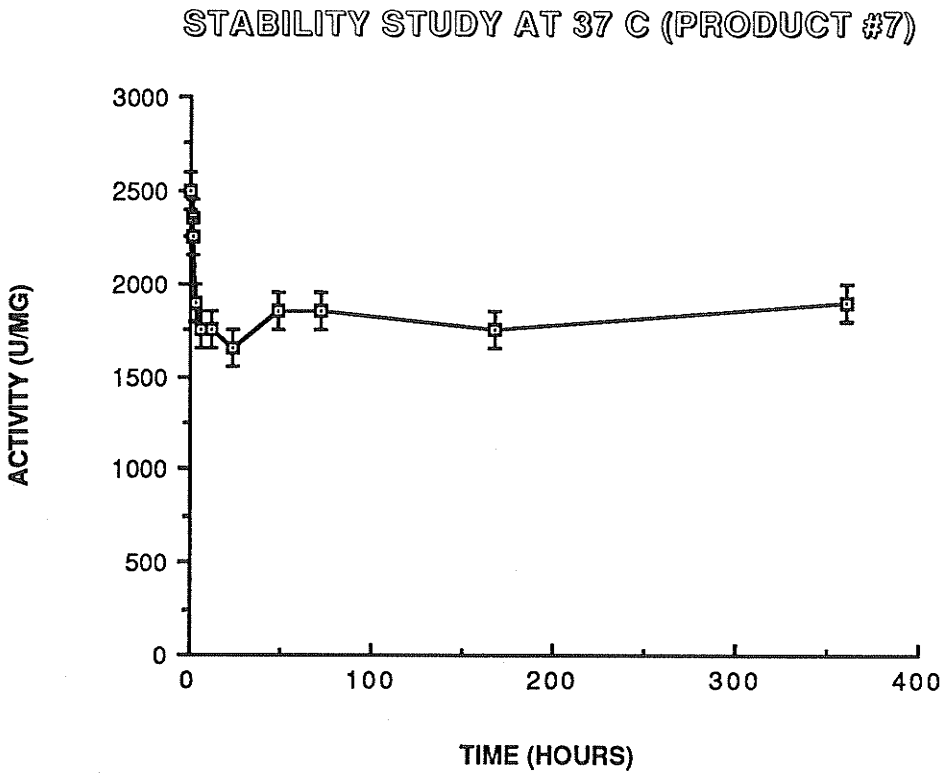


Figure 25

3.1.5. Stability Study at Room Temperature

In this study, the four formulations containing mannitol were used. Each vial was reconstituted with 1.0 mL sterile Water for Injection USP and then stored at room temperature (21 °C). Table 14 lists the superoxide dismutase activities of the four preparations at room temperature determined using an illumination time of 3 1/4 minutes.

Table 14. Activities of Superoxide Dismutase in Formulations Stored at Room Temperature

TIME(MONTHS)	PROD. 1	PROD. 3	PROD. 5	PROD. 7
0	2300	2300	2500	2300
	2400	2400	2400	2300
1	2500	2400	2400	2500
	2300	2500	2300	2300
2	2500	2400	2500	2300
	2300	2300	2500	2400
3	2200	2500	2500	2500
	2300	2500	2500	2300
4	2400	2400	2300	2400
	2400	2500	2300	2400
5	2500	2300	2500	2400
	2500	2400	2500	2500
6	2300	2300	2400	2400
	2400	2500	2500	2400

Table 15. Statistical Parameters of Superoxide Dismutase Activities For Stability Study at Room Temperature

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE
MODEL	27	260000.000	9629.630
ERROR	28	160000.000	5714.286
CORRECTED TOTAL	55	420000.000	
MODEL F=1.69			PR > F=0.0881
R-SQUARE	C.V.	ROOT MSE	Y MEAN
0.619048	3.1497	75.59289460	2400.000

SOURCE	DF	TYPE I SS	F VALUE	PR > F
TIME	6	35000.000	1.02	0.4323
FORMULA	3	32857.142	1.92	0.1498
TIME* FORMULA	18	192142.857	1.87	0.0669

STUDENT-NEWMAN-KEULS' TEST

ALPHA=0.05	DF=28	MSE=5714.29	
# OF MEANS	2	3	4
CRITICAL RANGE	58.5264	70.6927	78.0114
GROUPING	MEAN	N	PROD. #
MEANS WITH SAME LETTER ARE NOT SIGNIFICANTLY DIFFERENT			
A	2435.71	14	5
A	2407.14	14	3
A	2385.71	14	7
A	2371.43	14	1

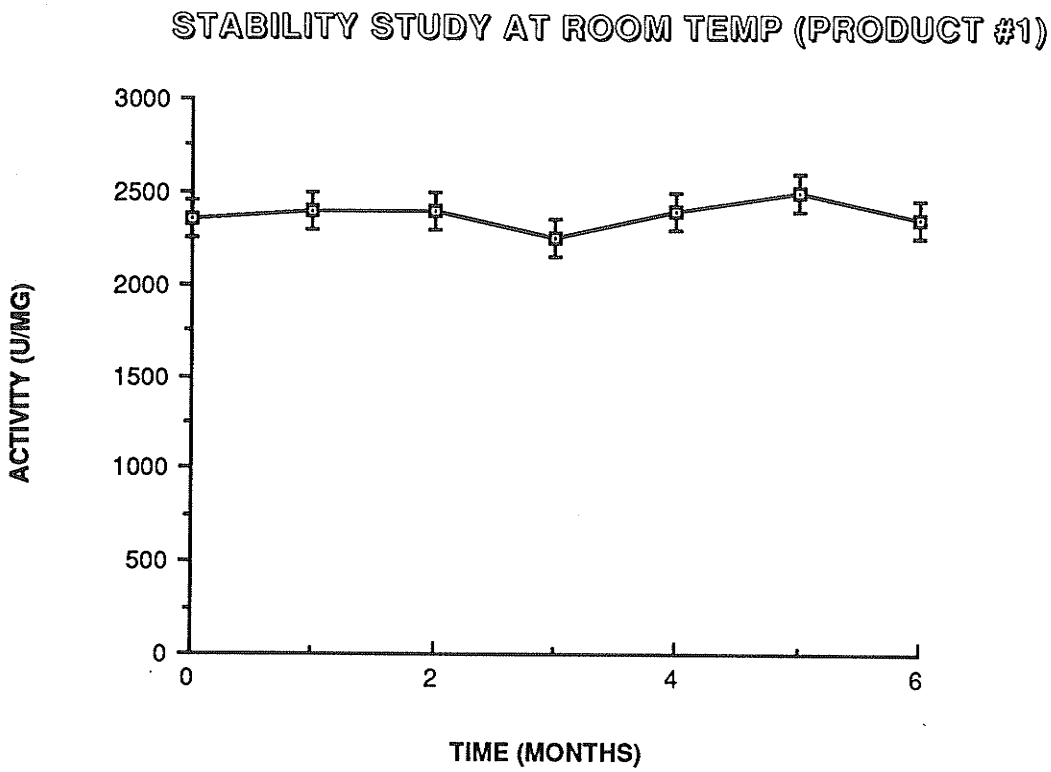


Figure 26

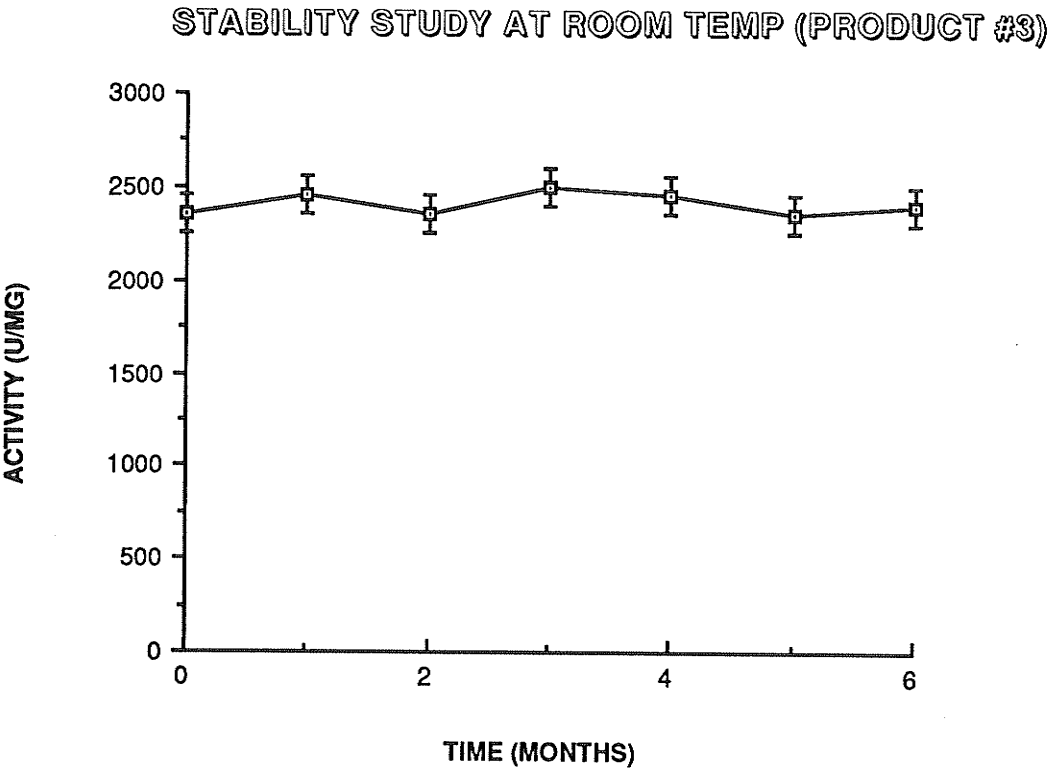


Figure 27

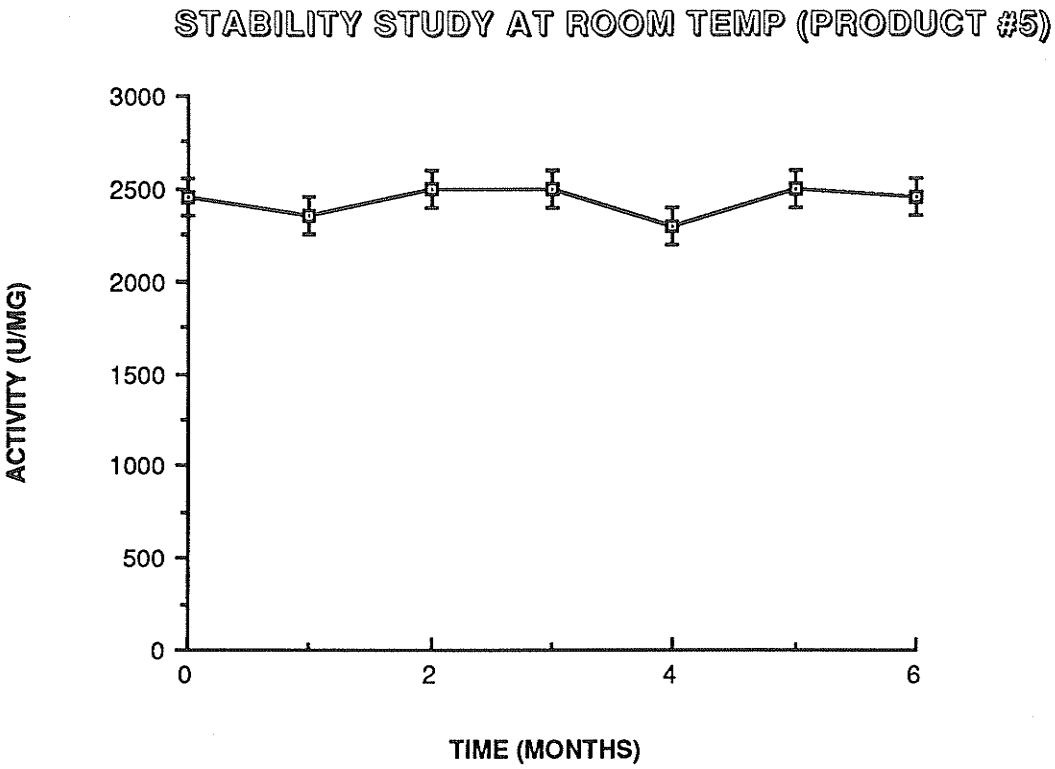


Figure 28

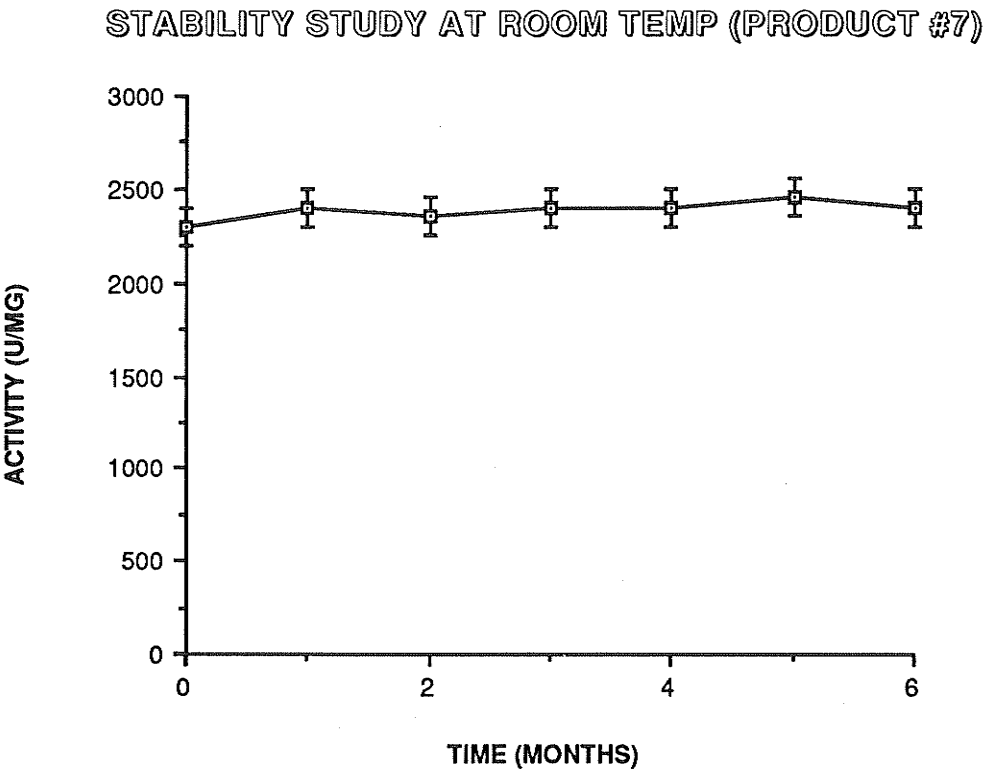


Figure 29

3.1.6. Stability Study at 4 ˚C

In this study, the four formulations containing the mannitol were tested for superoxide dismutase activity while being stored at 4 ˚C. Each vial was reconstituted with 1.0 mL sterile Water for Injection USP and then placed into the refrigerator. At the time of assaying, the preparations were allowed to warm up to room temperature. Table 16 lists the superoxide dismutase activities of the four preparations using an illumination time of 3 1/4 minutes.

Table 16. Activities of Superoxide Dismutase in Formulation Stored at 4 ˚C

TIME(MONTHS)	PROD. 1	PROD. 3	PROD. 5	PROD. 7
0	2500	2500	2300	2500
	2500	2400	2300	2400
1	2500	2500	2400	2400
	2400	2300	2500	2500
2	2400	2300	2300	2500
	2300	2400	2400	2500
3	2400	2400	2300	2400
	2400	2400	2300	2300
4	2500	2300	2500	2500
	2400	2500	2400	2400
5	2500	2500	2500	2500
	2400	2500	2500	2400
6	2400	2500	2300	2500
	2500	2400	2300	2400

Table 17. Statistical Parameters of Superoxide Dismutase Activities For Stability Study at 4 ˚C

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE
MODEL	27	203392.857	7533.069
ERROR	28	125000.000	4464.286
CORRECTED TOTAL	55	328392.857	
MODEL F= 1.69			PR > F=0.0875
R-SQUARE	C.V.	ROOT MSE	Y MEAN
0.619358	2.7614	66.81531048	2419.6428571

SOURCE	DF	TYPE I SS	F VALUE	PR > F
TIME	6	64642.857	2.41	0.0525
FORMULA	3	34821.428	2.60	0.0719
TIME*FORMULA	18	103928.571	1.29	0.2639

STUDENT-NEWMAN-KEULS' TEST

ALPHA=0.05	DF=28	MSE=4464.29	
# OF MEANS	2	3	4
CRITICAL RANGE	51.7305	62.4841	68.9530
GROUPING	MEAN	N	PROD. #
MEANS WITH SAME LETTER ARE NOT SIGNIFICANTLY DIFFERENT			
A	2442.86	14	7
A	2435.71	14	1
A	2421.43	14	3
A	2378.57	14	5

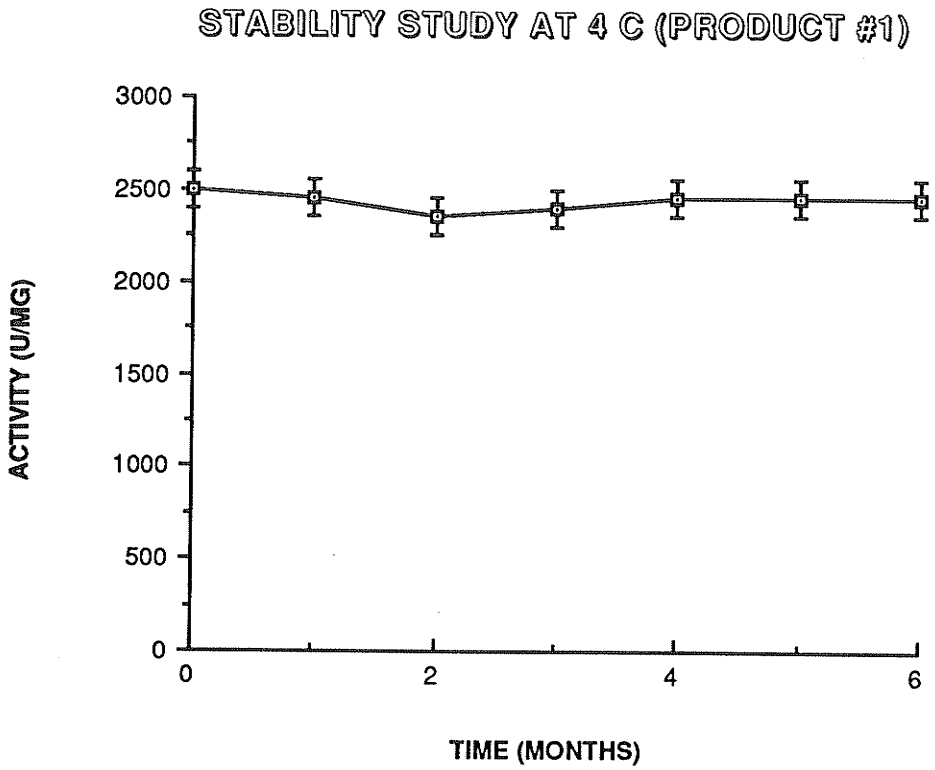


Figure 30

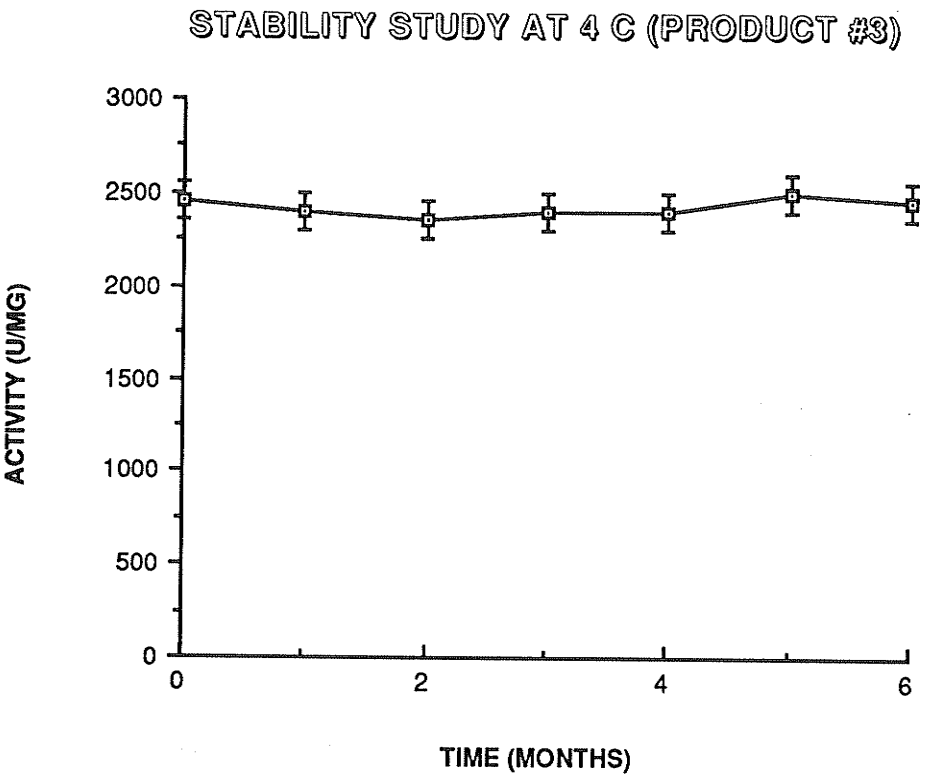


Figure 31

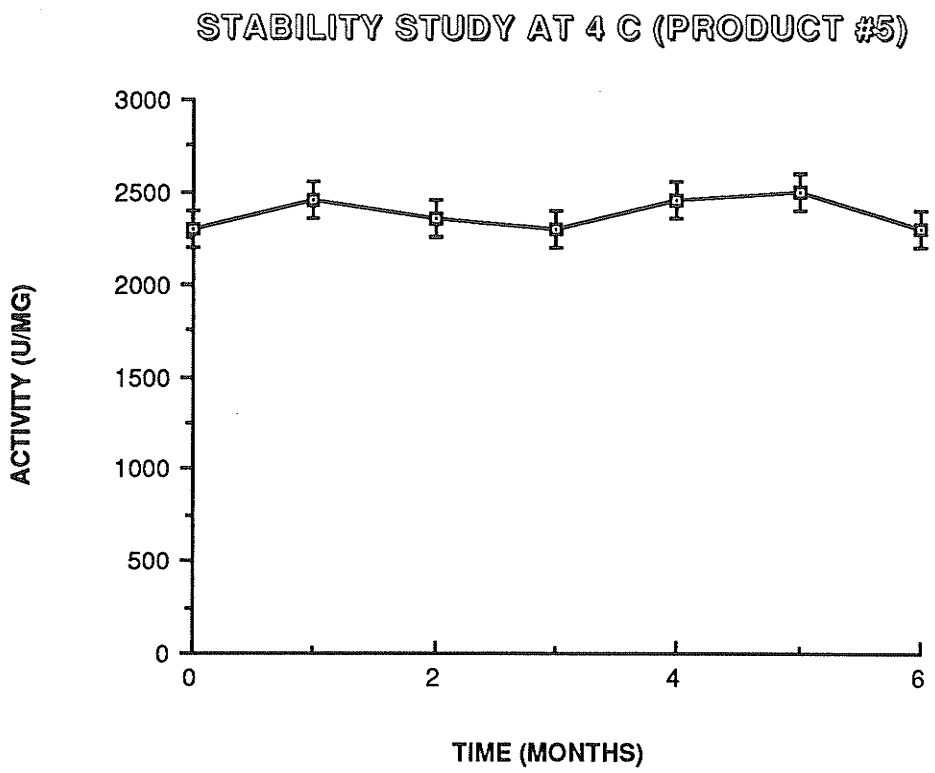


Figure 32

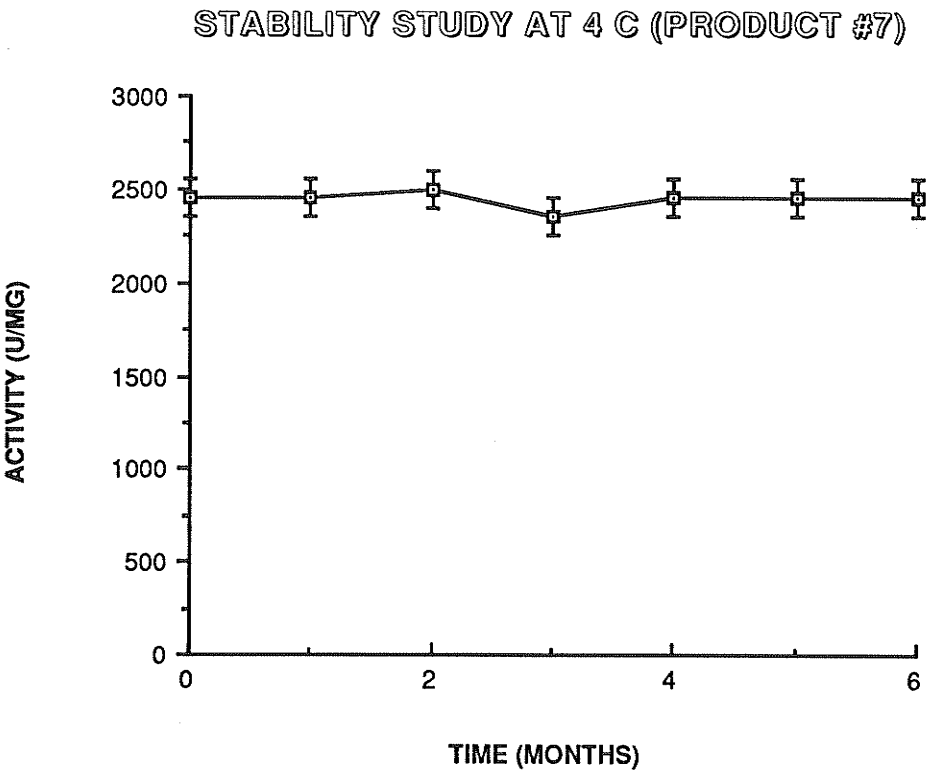
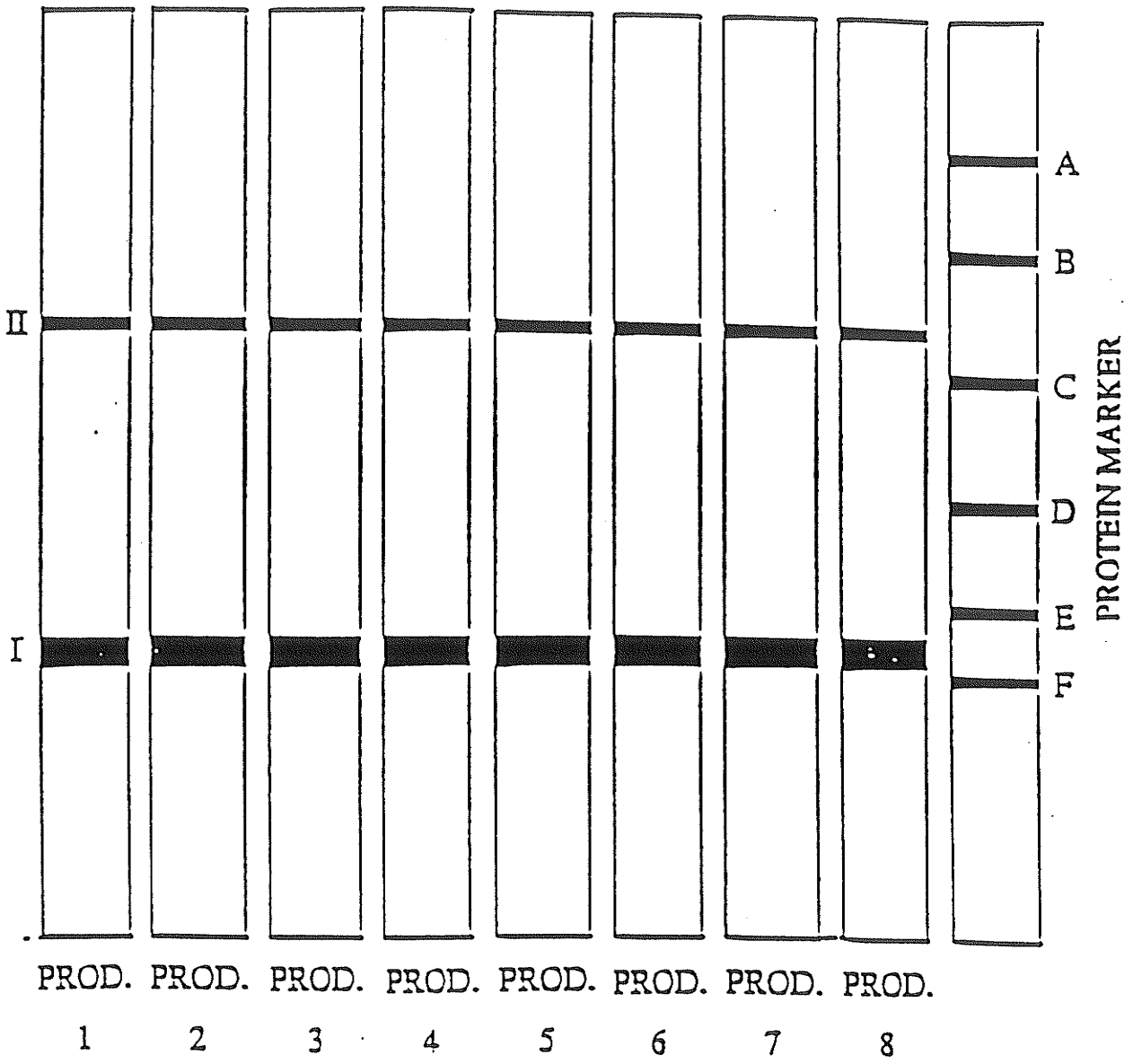


Figure 33

3.2. SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out on all eight formulations of superoxide dismutase. The time of electrophoresis coincided with the activity testing of SOD during the long term stability study, that is, at months 1, 2, 3, 5, 9, 10, and 12. Figure 34 illustrates an example of a typical electrophoretic gel depicting the eight formulations listed 1 through 8 along with the markers. As seen in figure 34, all of the eight formulations responded with the same relative mobility profile. Throughout the study period, there were no differences found in any of the gel samples.

Upon examination of the gels, a predominant band (I) indicates a Mr of about 18000 daltons. A less pronounced band (II) was also found on the gels equivalent to a Mr of about 55000 daltons.

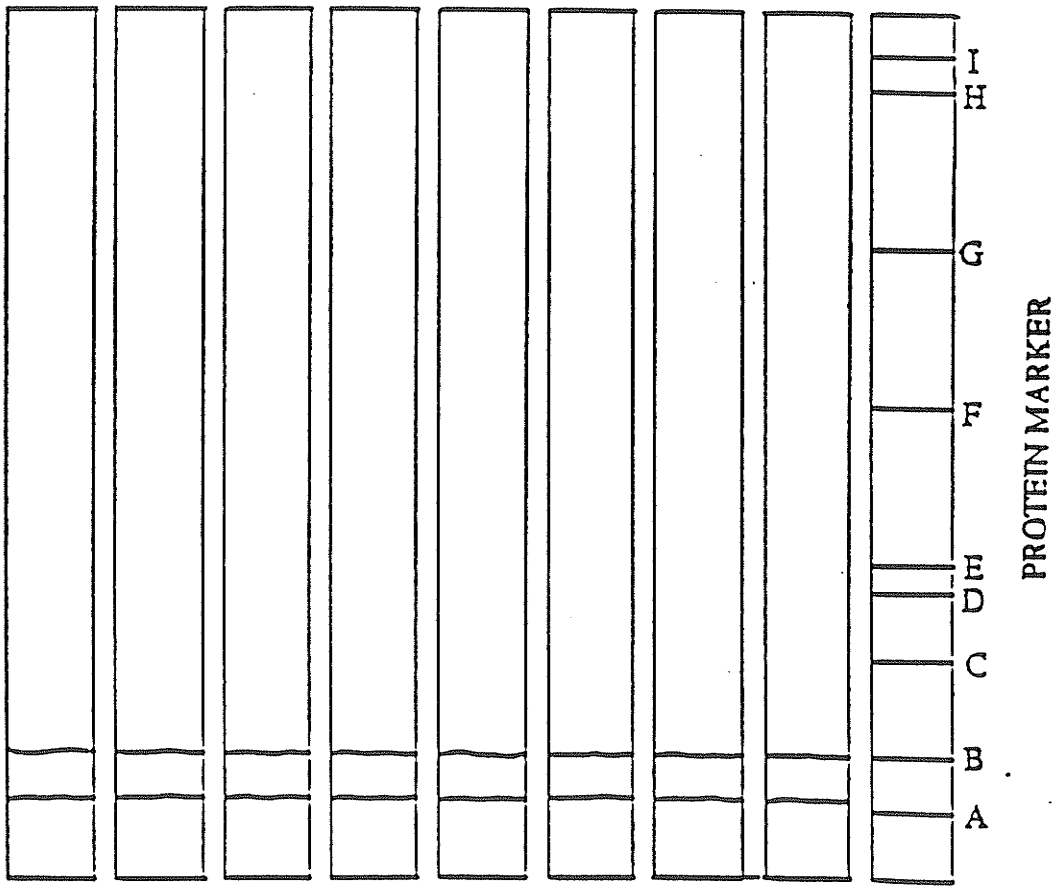


* Identification of Protein Markers as per Table 3 (pg. 50)

Figure 34 SDS-PAGE of Superoxide Dismutase in Formulation

3.3. Isoelectric Focusing

Isoelectric focusing was performed on all eight formulations as outlined in the methodology section. Isoelectric focusing was carried out upon initiation of the study as well as at the end of months 1, 2, 3, 5, 9, 10, and 12. The results of the isoelectric focusing remained constant throughout the one year period. Figure 35 illustrates typical results obtained from isoelectric focusing in the study. Two bands were continuously found for each product. The two bands corresponded to isoelectric points of 4.9 and 5.2.



* Identification of Protein Markers as per Table 4 (pg. 52)

Figure 35 Isoelectric Focusing of Superoxide Dismutase in Formulation

3.4. Fast Protein Liquid Chromatography

Figure 36 is a consistant FPLC printout of freshly reconstituted freeze dried superoxide dismutase in formulation. Table 18 shows the FPLC analysis breakdown of products 1 through 8.

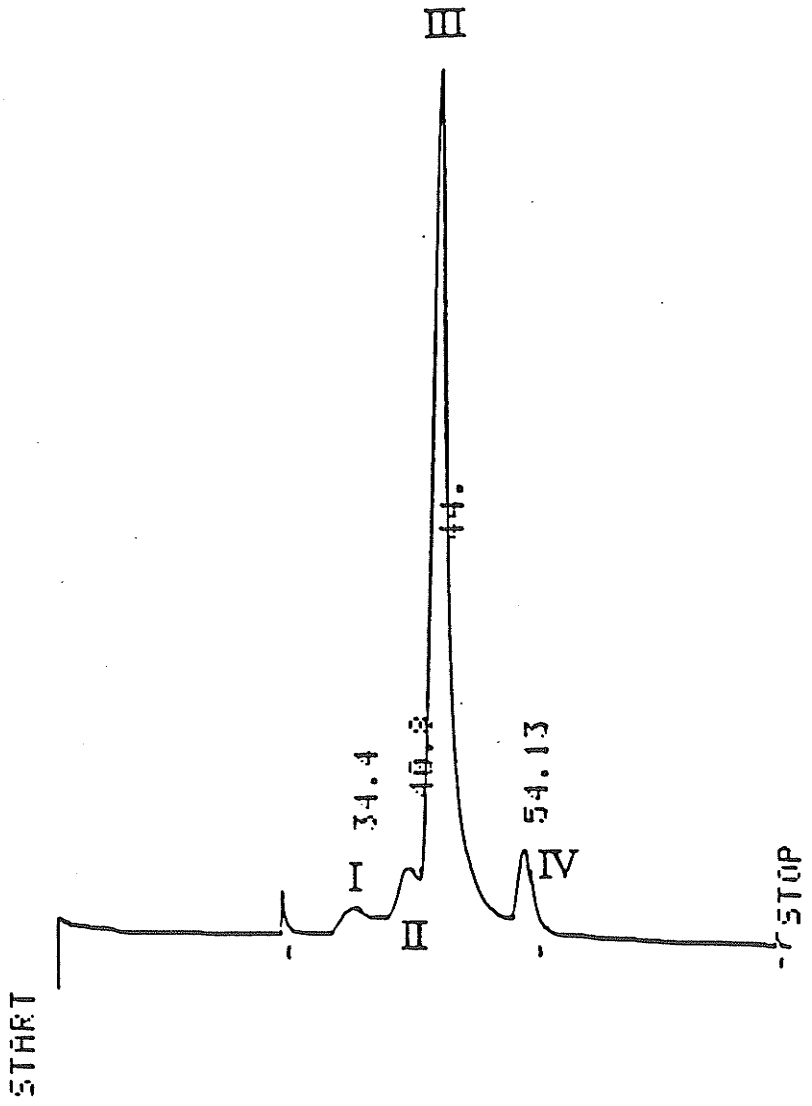


Figure 36 FPLC Run of Superoxide Dismutase in Formulation Freshly Reconstituted From a Freeze Dried Dosage Form

Table 18. FPLC Analysis of Products 1 through 8 Freshly Reconstituted From a Freezed Dried Dosage Form

PRODUCT #	PEAK	TIME (MINS)	Mr	RELATIVE CONC.
1	I	34.30	204200	3.5358
1	II	40.80	58800	8.5317
1	III	44.00	31600	79.6078
1	IV	54.13	4500	8.3245
2	I	34.41	204200	4.0835
2	II	40.81	58800	5.1896
2	III	44.01	31600	86.3091
2	IV	54.14	4500	4.4176
3	I	34.40	204200	4.6853
3	II	40.80	58900	7.0423
3	III	44.00	31600	84.2005
3	IV	54.13	4500	4.0716
4	I	34.41	204200	4.4396
4	II	40.81	58900	6.3986
4	III	44.01	31600	85.5300
4	IV	54.14	4500	3.6316
5	I	34.40	204200	4.4326
5	II	40.54	61700	6.1682
5	III	44.00	31600	84.9990
5	IV	54.14	4500	4.4002

Table 18 Cont'd				
6	I	34.14	213800	4.7370
6	II	40.01	68700	6.6466
6	III	44.01	31600	84.8142
6	IV	53.88	4600	3.8020
7	I	34.40	204200	3.8573
7	II	40.80	58800	4.4525
7	III	44.00	31600	78.8795
7	IV	53.86	4700	12.8106
8	I	34.14	213800	4.0137
8	II	*	*	*
8	III	44.01	31600	85.4909
8	IV	54.14	4500	10.4952

* NO PEAK FOUND

Figure 37 is a typical FPLC printout of products 1 through 8 after being reconstitute from a freeze dried state and then being stored at 4 ˚C for 9 months. Table 19 gives detailed results from each individual FPLC printout for each product.

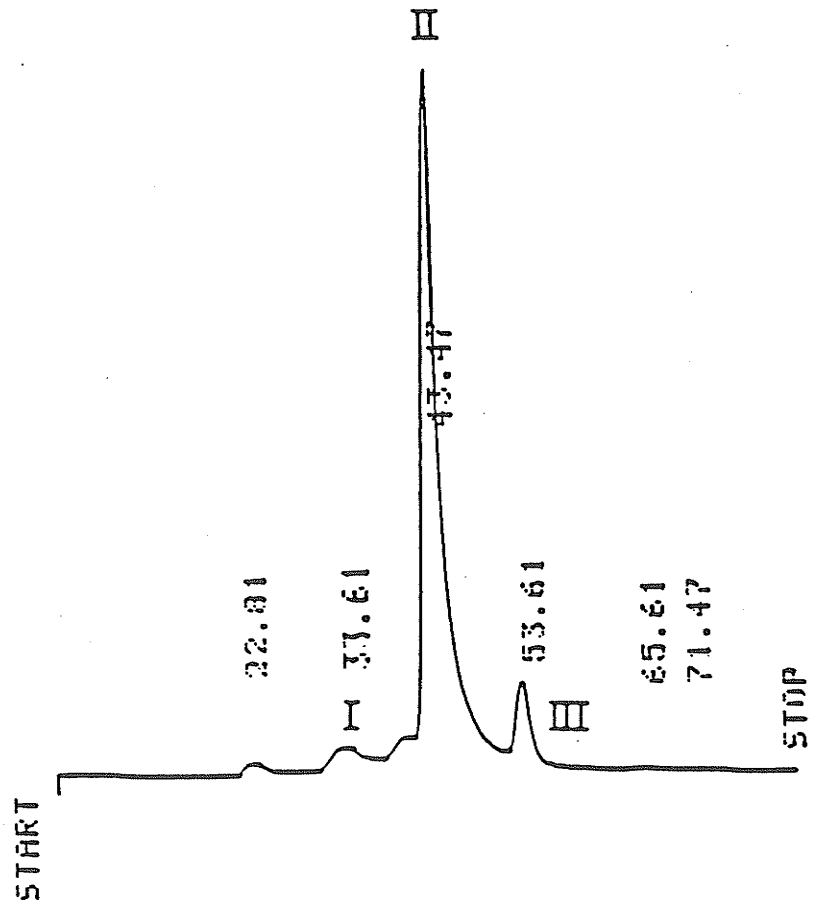


Figure 37. FPLC Run on Superoxide Dismutase in Formulation Stored at 4 ˚C for 9 Months After Being Reconstituted From a Freeze Dried Dosage Form

Table 19. FPLC Analysis of Products 1 Through 8 Stored at 4 ˚C After Initial Reconstitution From a Freeze Dried Dosage Form

PRODUCT #	PEAK	TIME(MINS)	Mr	RELATIVE CONC.
1	I	*	*	*
1	II	43.47	35000	85.3882
1	III	53.60	4900	14.6116
2	I	33.61	239900	4.2026
2	II	43.47	35000	86.8097
2	III	53.61	4900	8.9875
3	I	33.87	229000	4.9215
3	II	43.47	35100	86.1661
3	III	53.61	4900	8.9123
4	I	33.61	239900	6.0001
4	II	43.48	34700	84.6941
4	III	53.61	4900	9.3049
5	I	33.87	229000	4.7859
5	II	43.47	35100	85.4313
5	III	53.6	4900	9.7827
6	I	33.60	23900	4.0018
6	II	43.20	37000	87.1152
6	III	53.34	5100	8.8829

Table 19 Cont'd				
7	I	33.61	239900	3.6550
7	II	43.48	34700	78.4285
7	III	53.34	5200	17.9164
8	I	33.61	239900	4.0191
8	II	43.21	36900	79.5340
8	III	53.34	5100	16.4469

* NO PEAK FOUND

Figure 38 is representative of products 1 through 8 which have been kept frozen for 6 months after being reconstituted from a freeze dried state. Table 20 lists results obtained from the individual FPLC printouts of each product.

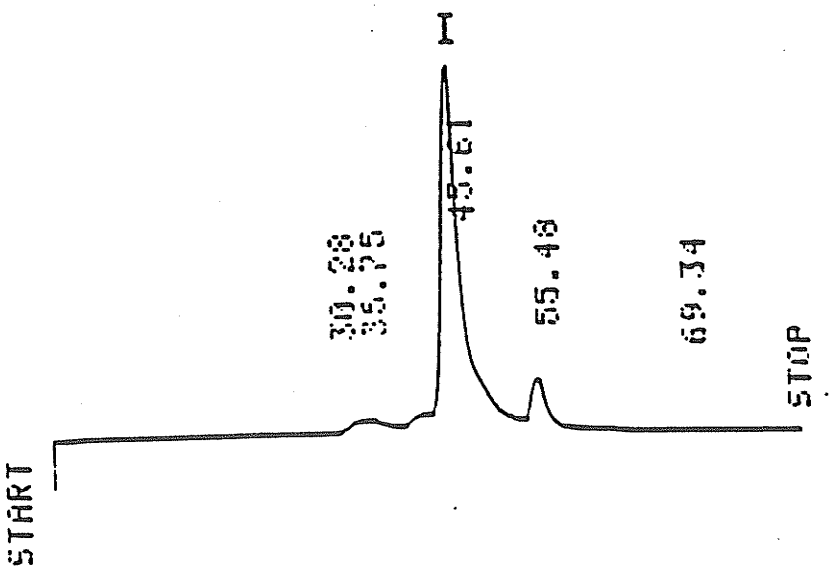


Figure 38. FPLC Run on Superoxide Dismutase in Formulation After Being Reconstituted and Then Refrozen for 6 Months

Table 20. FPLC Analysis of Products 1 Through 8 Kept Frozen After Initial Reconstitution From a Freeze Dried Dosage Form

PRODUCT #	PEAK	TIME(MINS)	Mr	RELATIVE CONC.
1	I	45.61	31600	85.6697
2	I	45.61	31600	84.4270
3	I	45.87	30200	86.1655
4	I	45.33	33600	92.5760
5	I	45.87	30200	84.4848
6	I	45.60	31600	92.8146
7	I	45.60	31600	77.2093
8	I	45.33	33600	78.6431

CHAPTER IV

DISCUSSION

4.1. Superoxide Dismutase Assays and Stability Studies

Upon initiation of this study, a reliable and easily reproducible assay had to be established. The assay choosen was based on the photoreduction of nitroblue tetrazolium(49). Nitroblue tetrazolium was utilized as a detector of the superoxide anion. Activity of superoxide dismutase was determined by its ability to inhibit the reduction of nitroblue tetrazolium due to superoxide. Reduction of nitroblue tetrazolium not involving superoxide does not interfere with this assay provided that the extent of such reaction pathways is not greater than that of the superoxide dependent pathway.

Illumination of the reaction mixture which contained methionine, riboflavin and nitroblue tetrazolium, produced a linear accumulation of formazan. Superoxide dismutase is without any effect in the absence of oxygen, however, in the presence of oxygen, superoxide dismutase inhibits the reduction of nitroblue tetrazolium(49). This leads to the speculation that photoreduced riboflavin can directly reduce nitroblue tetrazolium or reduce it via the superoxide anion acting as an electron-carrying intermediate.

In the present study the illumination of the reaction mixture resulted in an increase linear absorbance at 560 nm of about 0.060/minute. The assay procedure used had the added benefit of being readily standardized as well as being independent of other enzymes and proteins such as cytochrome c and xanthine oxidase which are used in alternative assays (47,55). By definition, one unit of superoxide dismutase results in a 50% inhibition in the reduction of nitroblue tetrazolium. Under these conditions, it was found to be equivalent to approximately 0.310 μg of bovine superoxide dismutase per millilitre. Through repeated trials, the maximal degree of inhibition produced by bovine superoxide dismutase was found to be about 85%. Higher degrees of inhibition may have been prevented as a result of an oxygen-independent mechanism resulting in the transfer of electrons from the photoreduced riboflavin to nitroblue tetrazolium. However, this does not present any problem in determining superoxide dismutase activity since a unit of superoxide dismutase activity is the amount required to produce half-maximal activity. Thus, the presence of any superoxide anion-independent routes for nitroblue tetrazolium reduction would not affect the determination of superoxide dismutase activity.

Standardization of the superoxide dismutase assay was required to obtain comparable results over the course of the study. This was established using a sample of bovine superoxide dismutase with a known activity of 3200 u/mg. After repeated trials, it was found that an illumination time of 3 1/4 minutes resulted in reproducible activities of 3200 u/mg. Examination of the statistical parameters of the 26 runs indicated a standard deviation of 138.03. This figure was rounded off to 100 and was used to set the error limits on the

activity studies. In other words, the error limits of this particular superoxide dismutase assay was set at +/- 1 standard deviation.

In studying the eight superoxide dismutase preparations stored at 4°C in the freeze dried state, it was found that there was no statistically significant change in activity throughout the one year study period. Statistically, no difference was found among the eight products with respect to formulation or stability. This suggests that in the freeze dried state, the stability of superoxide dismutase is not be dependent on formulation, under the conditions used in this study. From graphs 10 to 17 it is clear that the slight variations in activities are within the error limits of the assay.

In evaluating superoxide dismutase stability at 57 °C, four formulations were chosen. The four formulations were products 1, 3, 5, and 7 and were selected because of their mannitol content. As mentioned previously, mannitol was added to give structure or a matrix to the dried product. Upon initiation of the accelerated study at 57 °C, there was no statistical difference between the four formulations with respect to activity. The average activity of the four preparations at the start of the study was 2350 u/mg. The study period lasted 20 hours at which time the activities of products 1, 3, and 5 were less than 200 u/mg. Upon completion of this study a statistically significant difference in stability between the four products was detected. A Student-Newman-Keuls' test was performed and revealed that product 7 was statistically superior with respect to the other formulations, having a mean activity of 1445 u/mg. The order of decreasing stability of the remaining preparations was product 1, product 5, and product 3, having mean activities of 1385, 1265, and 1070 u/mg respectively. As revealed by the Student-

Newman Keuls' test, each formulation was statistically different from the other with respect to product stability. One major consideration that must be taken into account in this particular study is the fact that the loss of superoxide dismutase activity at 57 ˚C may have been the result of protein denaturation. Most proteins maintain their activity under narrow limits dependent on pH and temperature. At temperatures greater than 60 ˚C most globular proteins become denatured and as a result lose their biological activity(59). Thus, it is difficult to ascertain whether or not the native structure of the superoxide dismutase has been unfolded or become denatured at 57 ˚C. Perhaps differential scanning calorimetry studies could be performed to determine the degree of unfolding. Since biological activity is the result of native conformation which in turn is ultimately influenced by the amino acid sequence, denaturation by high temperatures or pH will result in an inactive protein. In this study it would appear that pH is not a critical factor in denaturation since other studies at room temperature display no loss of activity.

In a subsequent study, the evaluation of superoxide dismutase stability at 37 ˚C was determined. Products 1, 3, 5, and 7 were again chosen. Upon initiation of the study, the products were reconstituted and then maintained at 37 ˚C in the liquid state for 15 days. At the onset of the study there was no statistical difference between the four formulations with respect to activity. The average activity of the four products at the beginning of the study was 2400 u/mg. Examination of the data and statistics at the end of the study indicated that the four formulations differed significantly. A Student-Newman-Keuls' test was performed and revealed that product 7 had a statistically greater mean activity as compared with the other products. Also

the Student-Newman-Keuls' test showed that there was no statistically significant difference between the activities of products 1, 3, and 5. Therefore, based on this, product 7 appeared to be the superior product with a mean activity of 1950 u/mg. The measured decrease in activities of products 1, 3, and 5 was 41%, 47%, and 45% respectively. Since product 7 was superior in both of the previous studies, there is an indication that superoxide dismutase at 57 ˚C is not denatured.

Another stability study on the four products containing mannitol was also conducted at room temperature. In this study the products were reconstituted at the start of the study and were kept at room temperature as solutions for the duration of the study. The study period was six months. At the onset of this study the average activity of the preparations was 2350 u/mg. After six months of standing at room temperature there was no statistical difference between the four formulations with respect to activity. Thus, it is clear that at room temperature, superoxide dismutase in these particular formulations is very stable.

A final stability study was carried out at 4 ˚C. In this case, the four formulations containing mannitol were reconstituted with sterile Water for Injection USP at the start of the study and then stored for six months at 4 ˚C in the refrigerator. Samples were tested on a monthly basis for activity. Similar to the previous study at room temperature, there were no significant differences in activities either at the start of the study or after the six month duration of the study among the four formulations.

Based on all of the stability studies conducted on superoxide dismutase, the accelerated studies at 57 °C and 37 °C provided the most information. Both of these studies indicated that product 7 showed the best stability. Product 7 was formulated in a citrate buffer (20 mM) at pH 6.6 with 20 mg mannitol. Product 3, which had been formulated in a phosphate buffer (20 mM) at pH 7.8 with 20 mg mannitol did not to do very well. The reduced stability may be the result of the phosphate inhibiting superoxide dismutase activity. Monta de Freitas and Valentine conducted studies indicating the Arg-141 of superoxide dismutase is a binding site for phosphate and once phosphate binds to Arg-141, neutralization of the positive charge on the side chain of Arg-141 results(60). Once neutralized, this amino acid residue can no longer aid in the electrostatic guidance of the superoxide anion to the copper site on the enzyme. An alternate possibility of phosphate-dependent inhibition of superoxide dismutase is that phosphate binds directly with the active site. However, Monta de Freitas and Valentine found no visible or ESR spectral changes analagous to those found in solutions of CuZn-superoxide dismutase at high phosphate concentrations(60).

4.2. SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis is a widely employed technique used for determining the molecular weights of proteins. In this particular study, SDS-polyacrylamide gel electrophoresis was carried out as described in the experimental section.

In performing SDS-polyacrylamide gel electrophoresis, an understanding of how the constituent polypeptides bind is crucial in the characterization of

protein subunits. Abernethy *et al.* (61)have determined that the 2 polypeptide subunits of superoxide dismutase are not covalently joined but instead are held together by unusually strong noncovalent bonds. In their studies, the introduction of 2-mercaptoethanol prior to electrophoresis resulted in two distinct bands having molecular weights of approximately 16000 and 55000. It would appear that the molecular weight of the undissociated protein possesses a molecular weight of approximately 55000. This figure is larger than that calculated from the amino acid sequence as well as from that determined by physico-chemical procedures(61). This could be explained in part by the fact that incompletely denatured proteins, as a result of disulfide bonds or other structural features, may display anomalous sodium dodecyl sulphate binding. As a result anomalous molecular weight values on SDS-polyacrylamide gel electrophoresis may result.

In the present study, two bands consistently appeared, one with a molecular weight of approximately 18000 and the other with a molecular weight of approximately 55000. The band with molecular weight of 18000 would appear to represent the subunit structure of superoxide dismutase. A review of the literature indicated that the likely molecular weight of the bovine superoxide dismutase subunit structure to be about 16000(42,61,62,63). However, there are also published results showing that the subunit structure of human superoxide dismutase having a Mr between 18000 and 19000(58). The latter corresponds to the results obtained in this particular study. Although not consistent with the sequence data on the human enzyme, anomalous results using SDS-polyacrylamide gel electrophoresis have been documented previously(42,58,61,63,). According

to McCord and Fridovitch, the Mr of human CuZnSOD also appeared to be about 18000(47).

A second less predominant band found in the present study appeared at about 55000. This result is again supported by the literature(42,61,62,63). Two explanations have been developed for this band. As mentioned previously, Abernethy *et al.* attributed it to incomplete denaturation and anomalous binding to sodium dodecyl sulphate. Weser *et al.* on the other hand, suggests that it represents a tetrameric species of the subunit with a molecular weight of 16000(62).

Of interest in this study is that no band is detected at approximately 32000 representing the native erythrocuprein form of superoxide dismutase. This was consistent with the results of Weser *et al.*(62). Again this is probably the result of the presence of sodium dodecyl sulphate and the pretreatment with 2-mercaptoethanol.

The results obtained with SDS-polyacrylamide gel electrophoresis throughout the study period, revealed that there were no changes in the gels. This indicates that no alteration in the protein structure had occured over the one year period.

4.3. Isoelectric Focusing

Using the isoelectric focusing procedure, two bands were found for every preparation throughout the study period. The two bands represented isoelectric points of 4.9 and 5.2. These values reflect similar results by

previous investigators (64,65,66,67). The appearance of two bands indicated charge micro-heterogeneity as suggested by R.J. Weselake *et al.*(64). Micro-hetergeneity is not the result of carrier ampholytes interacting with superoxide dismutase since starch gel electrophoresis has also displayed micro-heterogeneity of human superoxide dismutase(66). As Weselake *et al.* indicated, these two bands represents the two charged isomers of human CuZnSOD(64).

4.4. Fast Protein Liquid Chromatography

Upon inspection of the FPLC output representing freeze dried products freshly reconstituted with 1.0 mL sterile Water for Injection USP, four peaks were consistently found. The major peak occurred at a time of approximately 44 minutes and corresponded to a molecular weight of about 31600. This peak represented the native superoxide dismutase molecule. As mentioned previously under the SDS-polyacrylamide gel electrophoresis results, this compound did not appear as a band on the gel probably as a result of the presence of sodium dodecyl sulphate and pretreatment with 2-mercaptoethanol in the SDS-polyacrylamide gel electrophoresis process.

Another peak which consistently occured on the FPLC output of the freshly reconstituted freezed dried products was found at a time of about 40.8 minutes. This peak represented a molecular weight of approximately 58800 and corresponded to band II of the SDS-polyacrylamide gel electrophroesis studies (fig 34) which had a determined Mr of about 56000. As mentioned previously, two possible explanations were given for the presence of this band: 1) The band of the SDS-polyacrylamide gel

electrophoresis and peak of the FPLC represents the tetrameric species of a subunit a with molecular weight of about 16000 and 2) incomplete denaturation and anomalous binding to sodium dodecyl sulphate. If the possibility of a tetrameric species is to be considered, one would expect the species to possess a molecular weight of approximately 64000. Since this study showed a consistent molecular weight between 56000 and 58800 it is likely that the second explanation of incomplete denaturation and anomalous binding to sodium dodecyl sulphate plays an important role in SDS-PAGE

.

Two other peaks found with the freshly reconstituted freeze dried products occured at times close to 34.4 and 54.1 minutes and gave molecular weight values of approximately 204200 and 4500 respectively. These peaks probably represent impurities found within the formulations. The FPLC procedure did not illustrate any peaks corresponding to the subunit species of superoxide dismutase as appeared on the electrophoretic gels. However, this is not surprising since there is no treatment of the superoxide dismutase samples with sodium dodecyl sulphate or 2-mercaptoethanol prior to the FPLC procedure.

FPLC analysis was also performed on products which were freshly reconstituted from the freeze dried state, and then either maintained at 4 ˚C for 9 months or refrozen for 6 months. All of the results showed peaks representing the native superoxide dismutase protein to be present. Of interest however, is that neither the solution kept at 4 ˚C or the frozen product gave a peak corresponding to a moleculr weight value of 58800. Lastly, it was found that the peak found at approximately 4500 was also

found in both the frozen products as well as the products kept at 4 ˚C in liquid state.

On tabulating the percent area under the FPLC curve of the native superoxide dismutase it was found that the freshly reconstituted freeze dried products had an average value for the dimeric species of 83.7289 with a standard deviation of 2.8415. The frozen products and the solutions kept at 4 ˚C had average values of 85.2500 and 82.5776 with standard deviatons of 5.6232 and 5.2538 respectively. The coefficient of variability using all values was 5.5594.

Thus by performing FPLC it would seem that the native superoxide dismutase species remained present and intact under the studied storage conditions. The main difference found between the three different states was that the peak at 58800 appeared only with the freshly reconstituted freeze dried product.

4.5. Overview of Study

In the present study, the choice of buffers or diluents was based on standard vehicles of existing parenterals. Citrate buffer, apart from being a standard vehicle is metabolized easily by the body. Phosphate buffer as well as NaCl are also standard vehicles with NaCl being the most widely used additive in parenteral dosage forms. Finally water was used as a control or internal standard.

The decision to produce freeze dried products resulted from preliminary studies on superoxide dismutase in solution which resulted in inadequate stability. Along with increased stability, a lyophilized product offers several other bennefits, including storage at room temperature for extended periods of time without the deterioration of the biological activity. As shown in this study all eight lyophilized products displayed no loss of activity throughout the one year study period. From the results obtained, one would expect any of the eight formulations to be adequately stable for an extended period of time. However, since product #7 exhibited greater stability at 57 ˚C and 37 ˚C it should be given greater consideration for further investigation. Such further investigation may include bioavailability studies, pharmacokinetic studies, animal studies and eventually human clinical trials.

Upon examination of the products, all of the formulations, excluding the superoxide dismutase formulated with NaCl, appeared as a white uniform cake. The products formulated with NaCl however, were slightly grey in color and had inferior textures. All eight products dissolved well when they were reconstituted.

Chapter V

CONCLUSION

The object of the present study was to prepare human superoxide dismutase in various formulations and conduct stability studies on them. At the start of the study, eight products of superoxide dismutase were formulated. Along with conducting stability studies on the various preparations, SDS-polyacrylamide gel electrophoresis, isoelectric focusing, and fast protein liquid chromatography analysis were also implemented confirming the stability of the enzyme in the various formulations.

The data collected throughout the one year stability study indicated that there was no statistically significant difference between the eight freeze dried formulatons stored at 4˚C with respect to activity. Results from SDS-polyacrylamide gel electrophoresis and isoelectric focusing reflected that the protein had not been altered during the study period.

Further investigation involving accelerated stability studies in solution at 57 ˚C and 37 ˚C were also performed. These studies revealed a statistically significant difference between the products with respect to activity. In both accelerated studies, product 7, formulated in a citrate buffer and mannitol, was shown to have the best stability.

Based on the results obtained from this study, product stability has been achieved by formulating superoxide dismutase in a freeze dried parenteral dosage form. Even though all of the eight freeze dried formulations displayed no loss of activity over the one year study period, product 7 was the product of choice since it maintained the greatest stability during the accelerated stability studies. As well as having the best stability, product 7 displayed pharmaceutical elegance along with good dissolution properties.

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APPENDIX

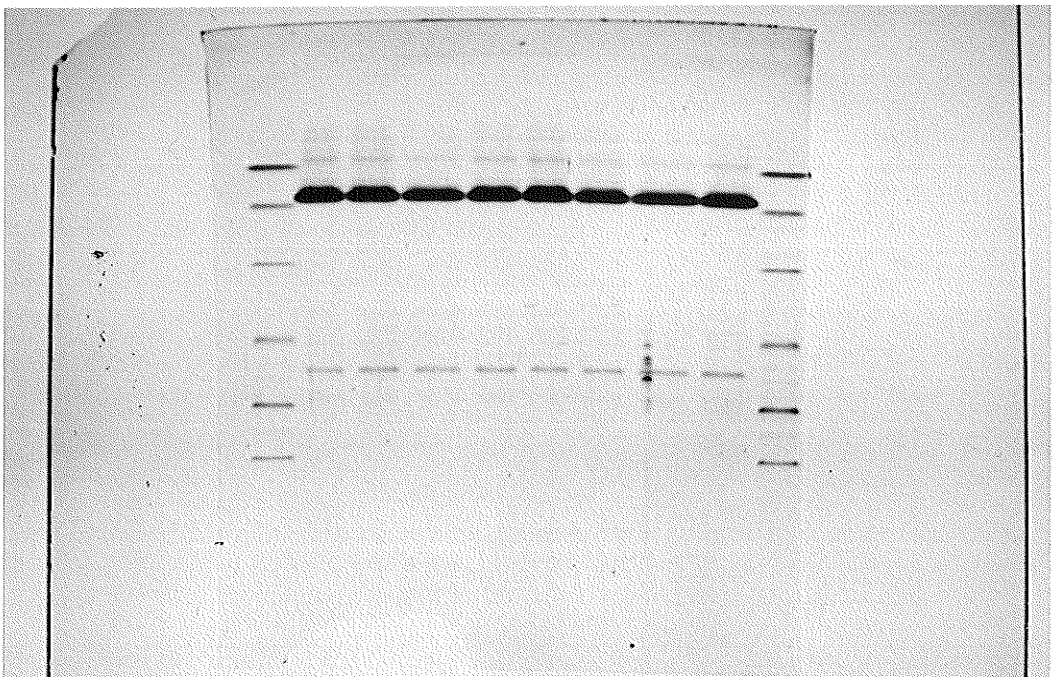

Figure 34

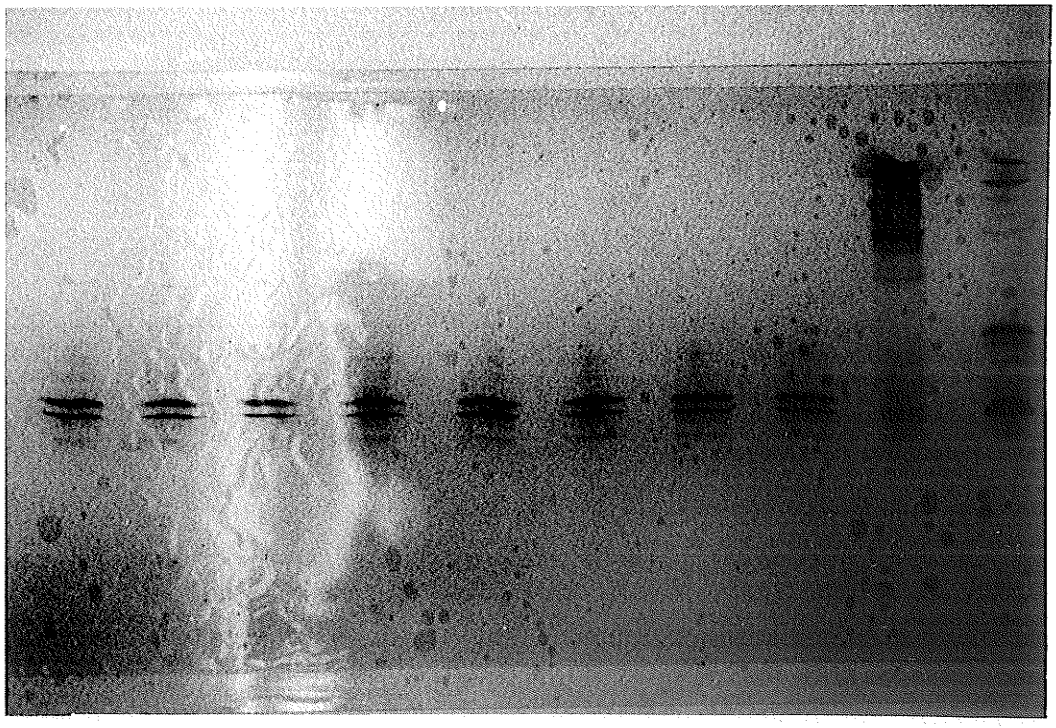

Figure 35