

Uricase Formulated with Polyethylene Glycol (Uricase-PEG 20): Biochemical Rationale and Preclinical Studies

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ABSTRACT. Objective. Humans have a non-sense codon inserted into the 5 prime end of the open reading frame of urate oxidase, and thus express an enzymatically inactive fragment of this enzyme; and consequently are unable to metabolize uric acid into allantoin and are prone to develop hyperuricemia and gout. Various urate oxidases (uricase) from mammals and microorganisms have been administered to humans with hyperuricemia and gout. Although successful in lowering plasma uric acid, these therapies have had limited application due to undesirable biochemical properties of the enzymes used, the short circulating half-life, and inherent antigenicity of these preparations.

Methods. We compared urate oxidase from a variety of sources for specific enzyme activity, pH optimum, affinity, and retention of enzyme activity under physiological conditions. A variety of polyethylene glycols (PEG) were tested to formulate uricase.

Results. Urate oxidase from *Candida utilis* had more favorable enzymatic properties and PEG of 20,000 MW (termed uricase-PEG 20) had greatly reduced antigenicity and increased circulating half-life as compared to those previously described.

Conclusion. It is anticipated that uricase-PEG 20 may have utility as a treatment for hyperuricemia and gout. (J Rheumatol 2002;29:1942-9)

Key Indexing Terms:

URICASE

URIC ACID

GOUT

URATE OXIDASE

Hyperuricemia results in gouty arthritis and chronic renal disease. Acute elevations of uric acid following cancer chemotherapy (tumor lysis syndrome) may result in acute uric acid nephropathy with resultant metabolic disorders and death¹⁻⁴. Treatment for these disorders is needed.

Uricase (urate oxidase, EC 1.7.3.3.) degrades the poorly soluble uric acid (~11 mg/100 ml H₂O), into the more soluble product allantoin (~147 mg/100 ml H₂O)¹. However, humans, chimpanzees, orangutans, and gibbons have a non-sense codon inserted into this gene that results in the synthesis of a short (10 amino acid) fragment devoid of enzymatic activity². As a result, high concentrations of uric acid can occur in humans, and when saturation is reached, this precipitates out of solution and collects in tissues and joints, causing a profound inflammatory reaction resulting in pain and loss of function and permanent damage to joints, connective tissues, and kidneys.

Individuals at risk of developing hyperuricemia include those undergoing cancer chemotherapy and organ transplant. Rapid destruction of tumors causes a release of large

amounts of uric acid into the blood, a disease known as tumor lysis syndrome, which is characterized by acute uric acid nephropathy and renal failure³⁻⁸. Transplant recipients develop hyperuricemia, which may result from poor kidney filtration complicated by the immunosuppressive therapies (e.g., cyclosporine) these individuals receive⁹⁻¹².

Investigators have administered uricase from a variety of microorganisms and animals to patients. These treatments with native uricase have been shown to decrease serum uric acid concentrations and to be effective in the treatment of hyperuricemia and gout, and serve as useful prophylaxis and treatment for hyperuricemia accompanying tumor lysis¹³⁻¹⁶. Native uricase from *Aspergillus flavus* is available for use in France and Italy (UricozymeTM, Sanofi-Synthelabo, Paris, France)^{14,15}, and a trial of native, nonrecombinant *Aspergillus* uricase has been performed in the USA¹³. Recombinant *Aspergillus* uricase, biosynthesized in the yeast *Saccharomyces cerevisiae* (rasburicase, Sanofi-Synthelabo), has also been tested in patients^{6,16-18}. Because humans do not make urate oxidase, all of these enzymes are highly antigenic and repeated administration of native uricase often results in allergic reactions, anaphylaxis, and even death^{6,10,11,13,16-19}. In addition, recombinant *Aspergillus* uricase biosynthesized in *S. cerevisiae* often hyperglycosylates using a nonmammalian stereochemistry, thus enhancing the immunogenicity of the recombinant protein. Thus a method to modify urate oxidase to decrease its immunogenicity has been recommended^{11,13}.

Covalent attachment of polyethylene glycol (PEG) to a number of therapeutic proteins has been shown to reduce

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their antigenicity and prolongs their circulating half-life^{20,21}. Some investigators used PEG of 5000 molecular weight to formulate uricase from *Candida utilis*²² and *Arthrobacter protoformiae*²³, and found these preparations were safe and effective in humans. There are many PEG available with varying effects on the antigenicity, immunogenicity, and half-life of a protein^{20,21}. We used a 20,000 MW PEG and a succinimidyl succinimide linker, known for its safety record in humans, to produce a formulation we have termed Uricase-PEG 20.

MATERIALS AND METHODS

Uricase isolated from *Arthrobacter* and hog liver was obtained from Sigma, St. Louis, MO, USA.

Production of recombinant uricase. The gene encoding uricase was isolated from genomic DNA purified from *C. utilis* (ATCC 9950; American Type Culture Collection, Bethesda, MD) by polymerase chain reaction (PCR). Recombinant uricase was produced in *Escherichia coli*, strain DG101 (ATCC 47041).

E. coli were grown to OD_{600nm} = 8.0 (which required ~6 h). Isopropyl β-D-thiogalactopyranoside (IPTG) (1 mM final concentration) was then aseptically added to the fermentor and the fermentation allowed to continue for 2 additional h. The cells were then collected by centrifugation (8000 × g for 10 min) and transferred to plastic storage containers, then stored at -70°C. A single 20 l fermentation produced 0.5 to 0.6 kg of cell paste.

The *E. coli* cells were lysed using a microfluidizer. The insoluble material (cell debris) was removed from the soluble material (which contains virtually all the uricase) by centrifugation (8000 × g for 10 min). The clarified cell lysate was mixed with ammonium sulfate (16.4 g per 100 ml of supernatant) and then passed through a 0.1 μm hollow fiber filter. The filtrate was then mixed with additional ammonium sulfate (21.4 g per 100 ml of filtrate) and the precipitated uricase was collected using a 100 kDa filter. The filtrate was then diafiltered using a 10 kDa cutoff hollow fiber filter with 5 volumes of 20 mM sodium phosphate buffer, pH 8.5.

The filtrate from the preceding step was applied to an anion exchange column containing Poros HQ resin (Perseptive Biosystems, Boston, MA, USA) equilibrated with 20 mM sodium phosphate buffer, pH 8.5. The column was then washed with 1 column volume of equilibration buffer. The uricase did not bind to the column under these conditions.

The flowthrough from the anion exchange column was then applied to a hydroxyapatite column preequilibrated with 20 mM sodium phosphate buffer, pH 8.5, and eluted with 200 mM sodium phosphate buffer, pH 8.5. This results in a preparation that is a single band on a sodium dodecyl sulfate-polyacrylamide electrophoresis gel with a specific enzyme activity of ~10 μmol/min/mg of protein. About 8 g of pure urate oxidase was obtained from a single 20 l fermentation.

The recombinant uricase is freely soluble at physiological pH in phosphate buffer and retains full enzyme activity for > 2 years when stored at 4°C. Alternatively, it may be frozen at -20°C or -70°C for many months and is stable for > 4 freeze-thaw cycles.

Formulation of bulk uricase with PEG. Purified uricase in elution buffer (200 mM sodium phosphate buffer, pH 8.5) was formulated with methoxy-SS-polyethyleneglycol MW 20,000 (PEG 20) (Shearwater, Huntsville, AL, USA) at a ratio of PEG to uricase of 30:1 (wt/wt). The PEG 20 was added to the uricase solution and allowed to stir for 2 h at room temperature. Unreacted PEG (PEG not covalently attached to uricase) was removed by diafiltration using 10 volumes of formulation buffer and a 30,000 MW cutoff hollow fiber filter cartridge (Microgon, Boston, MA, USA). The resulting material was termed bulk uricase-PEG 20.

Determination of PEG number. The trinitrobenzenesulfonic acid (TNBS) assay was used for determining the number of primary amines with a polyethylene glycol (PEG) attached (pegylated) as described by Davis, *et al*²².

Enzyme assay. Uricase activity was quantified using a uric acid diagnostic kit (Sigma). The specific activity of the enzyme is determined by incubating the plasma with uric acid and monitoring for the production of hydrogen peroxide using 4-aminoantipyrine and 3,5-dichloro-2-hydroxybenzenesulfonate and monitoring the absorbance at 520 nm. The amount of hydrogen peroxide formed is determined by comparison with standards containing known amounts of hydrogen peroxide. In the assay:

$$\text{Specific enzyme activity} = \frac{\mu\text{mol hydrogen peroxide produced}}{\text{min/mg protein}}$$

Enzyme activity is expressed in IU/ml; 1 IU is defined as the amount of enzyme that produces 1 μmol hydrogen peroxide/min.

Pharmacokinetics. The pharmacodynamics of uricase-PEG 20 were determined by measuring the amounts of plasma uric acid and allantoin using high performance liquid chromatography as modified²².

Determination of antibody titer. Levels of anti-uricase antibody were determined using an ELISA method. This method consists of using plastic 96 well polystyrene microtiter plates (Fisher Scientific, Pittsburgh, PA, USA) coated with a solution of uricase (0.3 mg/ml) in Tris buffered saline (TBS) (100 mM Tris, pH 8.0, and 150 mM NaCl) for 4 h. The plates were then rinsed twice and a solution of 50% fetal calf serum (FCS; Gibco, Grand Island, NY, USA) in TBS was applied for an additional 4 h. Next, the plates were rinsed and serial dilutions of the plasma (in 50% FCS and TBS) were added to the wells. The plates were allowed to stand 1 h at room temperature then rinsed 5 times with TBST (150 mM NaCl, 100 mM Tris, pH 8.0, and 0.05% Tween). Next, alkaline phosphatase antibody, rabbit anti-mouse IgG or IgE (Jackson Immunological Research, Bar Harbor, MA, USA) diluted 1:1000 in 50% FCS, and TBST was applied to the wells for 1 h. The wells were then washed 5 times with TBST and 3 times with TBS. A solution of p-nitrophenyl phosphate (Zymed, San Francisco, CA, USA) was then added to the wells and the plates incubated for 60 min at 37°C. Absorbance of the wells was determined at 405 nm.

The titer of the anti-uricase antibody was defined as the greatest dilution of the plasma that resulted in an absorbance of 2 times the background. Background was defined as the absorbance of wells without plasma.

Assay for neutralizing activity. To determine if the immune response was neutralizing, plasma was incubated with uricase-PEG 20 and the enzyme activity of the mixture was determined. An equal volume of undiluted plasma (0.01 ml) was mixed with an equal volume of 0.01 ml, of Uricase-PEG 20. The mixture was incubated 1 h at room temperature, then added to 1 mL of uric acid in sodium phosphate buffer (pH 7.4) and the amount of enzyme activity determined.

SDS-PAGE. Gels were run under reducing condition using precast polyacrylamide gels (10–20% gradient gels) (Novex, San Diego, CA, USA). Standards were also obtained from Novex (Mark 12 wide range molecular weight standards). Gels were stained with Coomassie brilliant blue (Pierce, Rockford, IL, USA).

Determination of protein concentration. The protein concentration was determined by the Bradford method with a BioRad kit (BioRad, Richmond, CA, USA) used as suggested by the manufacturer. A standard curve was constructed using known amounts of bovine serum albumin.

Toxicity testing in mice. Mice toxicity testing was performed by Covance Laboratories, Vienna, VA, USA.

RESULTS

Biochemical characterization of recombinant urate oxidase from *C. utilis*. Because humans do not express urate oxidase², all urate oxidases, irrespective of the species from which they are derived, are highly immunogenic. Thus we used urate oxidase from *C. utilis*, as this enzyme has the

highest affinity for uric acid and the greatest catalytic rate at physiological pH.

Urate oxidase was cloned from *C. utilis* and expressed in *E. coli*²⁴. The recombinant protein was very stable and freely soluble at physiological pH, unlike mammalian urate oxidases, which are unstable and sparingly soluble below pH 9. The recombinant enzyme was purified by column chromatography and was > 99% pure as determined by SDS-PAGE (Figure 1).

The recombinant uricase was then assayed for its ability to hydrolyze uric acid *in vitro*. A variety of other urate oxidases, all used previously in human clinical trials, were obtained and assayed using identical assay conditions (2 mg/100 ml uric acid, 100 mM phosphate buffer, pH 8.3, 24°C for 30 min) (Table 1). Note that 1 IU of enzyme activity is defined as that amount of enzyme required to convert 1 μ mol uric acid into 1 μ mol allantoin in 1 min at 24°C.

Subcellular localization studies indicate urate oxidase is a peroxisomal enzyme²⁵. Peroxisomes are intracellular organelles known to have high pH. All urate oxidases characterized to date have a basic pH optimum for maximal

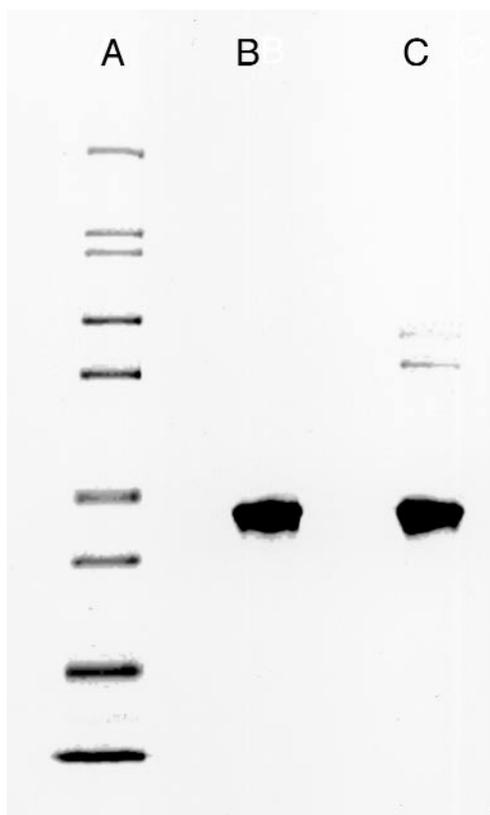


Figure 1. SDS-PAGE comparison of urate oxidase purified from *C. utilis* and recombinant *C. utilis* urate oxidase expressed in *E. coli*. Lane A: molecular weight standards (starting at top: 200, 116.3, 97.4, 66.3, 55.4, 36.5, 31, 21.5, 14.4 kDa). Lane B: recombinant urate oxidase from *C. utilis*, expressed in *E. coli*. Lane C: commercial *C. utilis* urate oxidase.

Table 1. Specific enzyme activity (SEA) of several urate oxidase enzymes. SEA of the enzyme is expressed in IU/mg of protein, where 1 IU is defined as that amount of enzyme that converts 1 μ mol of uric acid into 1 μ mol of allantoin and hydrogen peroxide in 1 min. Recombinant *C. utilis* enzyme has higher SEA compared to other uricases. All assays were performed at pH 8.3 to facilitate direct comparison of uricase-PEG 20 to published data on other uricases at that pH.

Reference	Source	Species	SEA
Uricase-PEG 20	Recombinant	<i>Candida utilis</i>	10
Davis ²²	Naturally produced	<i>Candida utilis</i>	3
Chua ²³	Naturally produced	<i>Arthrobacter protoformiae</i>	8
Uricozyme TM	Naturally produced	<i>Aspergillus flavus</i>	8.5
London ³⁴	Naturally produced	Hog liver	0.5

enzyme activity. It is intended that this enzyme will be administered to humans and catalyze hydrolysis of plasma uric acid. Thus, it was considered desirable that the activity of each of the enzymes be determined at various pH. To do this, enzyme was incubated in buffer (100 mM at pH indicated) with 2 mg/100 ml uric acid. An incubation temperature of 24°C and a reaction time of 20 min were used for all assays. The results are illustrated in Figure 2. All uricases tested had an alkaline pH optimum, as would be expected of an enzyme that is primarily localized to a peroxisome. However, the amount of enzyme activity each enzyme had at physiological pH of 7.4 was very different. The amount of enzyme activity of each of these enzymes at pH 7.4 is shown in Table 2.

Recombinant urate oxidase from *C. utilis* has much greater enzyme activity at physiological pH of 7.4. Thus it would be anticipated that less *C. utilis* urate oxidase protein would have to be administered in order to catalyze the hydrolysis of any given amount of uric acid in the plasma of humans compared to the other urate oxidases.

The affinity of each of these enzymes was also determined by reacting the enzyme with increasing concentrations of uric acid. Uricase from *C. utilis* has an optimal V_{max} and K_m compared to uricase from other sources (Table 3).

Preclinical studies with uricase-PEG 20. To reduce the immunogenicity and increase the circulating half-life of this enzyme, we formulated it with PEG in a similar manner as had been done previously²², with minor modification. Davis, *et al*²² and Chen, *et al*²⁶ first attached PEG to uricase, purified from *C. utilis*, using a succinimidyl succinimide chemical linker; this linker attaches PEG to the primary amines of the protein. They reported that attachment of PEG 5000 MW to roughly 50–70% of the primary amines successfully masked the immunogenicity of uricase in both mice and humans^{22,26,27}. Later it was shown that attachment of PEG 5000 MW to 60% of the primary amines of uricase isolated from *Arthrobacter protoformiae* also blocked the immunogenicity in humans²³.

We attached PEG of various molecular weights to recom-

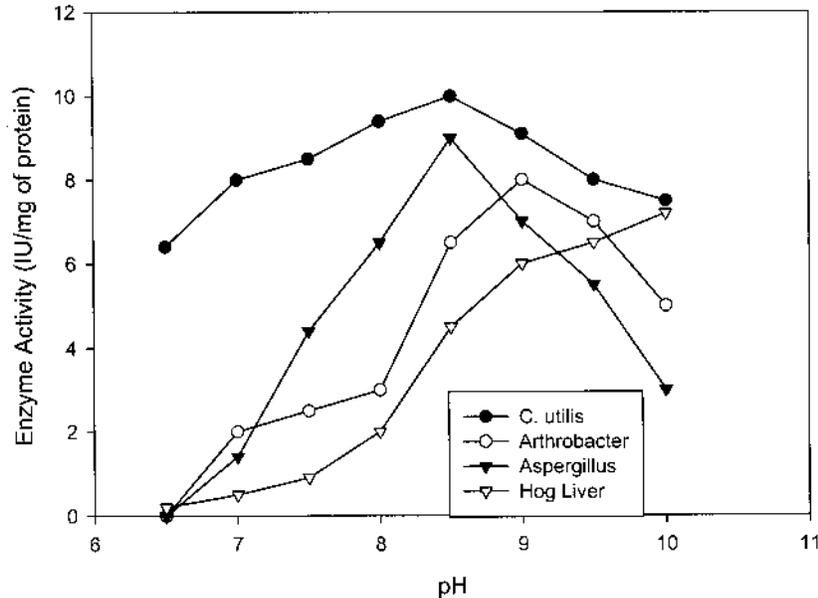


Figure 2. Effect of pH on enzyme activity of several urate oxidase enzymes. Urate oxidases from the indicated species were incubated with uric acid in 100 mM buffer (sodium phosphate pH 6.5–8.0 and sodium borate pH 8.5–10). Enzyme activity was determined by diminution of absorbance at 293 nm (the absorbance maximum of uric acid). Enzyme activity is expressed as IU of activity per mg protein. 1 IU is defined as that amount of enzyme activity needed to convert 1 μ mol uric acid into 1 μ mol allantoin in 1 min. The pH optima were determined for uricase from *Candida*, *Arthrobacter*, and hog liver. Data on *Aspergillus* (UricozymeTM) was obtained from the literature¹³.

binant uricase from *C. utilis*. These formulations were systematically tested for retention of specific enzyme activity and circulating half-life in mice. It was found that formulation with PEG of 20,000 MW was best, retaining more specific enzyme activity and enhancing circulating half-life. Figure 3 shows an SDS-PAGE comparison of native recombinant uricase from *C. utilis*, the same enzyme formulated with PEG 5000 MW used by Davis, *et al*²² and Chen, *et al*²⁶, and this same enzyme formulated with PEG 20,000 MW. Note that although the number of PEG molecules attached was similar for both molecular weights of PEG used, the specific enzyme activity was greater with

Table 2. Retention of specific enzyme activity (SEA) of various urate oxidases at physiological pH 7.4. The amount of enzyme activity at pH 7.4 was determined using the standard assay conditions of 2 mg 100 ml uric acid in 100 mM sodium phosphate, pH 7.4. The amount of enzyme activity observed at pH 7.4 was also compared with the amount of enzyme activity observed at the pH optimum for each individual enzyme and expressed as a percentage of the optimal activity.

Urate Oxidase Source	SEA at pH 7.4	Percentage SEA at pH 7.4
<i>Candida utilis</i>	8.2	82
<i>Arthrobacter protoformiae</i>	2.0	25
<i>Aspergillus flavus</i>	4.4	55
Hog liver	0.4	7

PEG 20,000 MW compared to PEG 5000 MW (8.6 and 5 IU/mg, respectively). Recombinant native *C. utilis* uricase has a specific enzyme activity (SEA) of \sim 10 IU/mg protein. Thus pegylation of *C. utilis* uricase with PEG 20,000 MW results in little change to the SEA (\sim 10 to 8.6 IU/mg), in contrast to PEG 5000 MW (\sim 10 to 5 IU/mg).

Pharmacokinetic studies of uricase-PEG 20 in mice. To determine the pharmacokinetics of uricase-PEG 20, we used mice as an experimental model system. In these experiments mice (4 per sex) were injected with uricase-PEG 20 using the intramuscular route of administration. The amount of uricase enzyme activity was determined at the indicated times and the means of the 4 mice in each group is shown in Figure 4. These data indicate that the pharmacokinetics of uricase-PEG 20 were nearly the same irrespective of the sex

Table 3. Michaelis-Menton constants of various urate oxidase enzymes. Analysis of the kinetics of the various urate oxidase enzymes was determined by incubation enzymes with various concentrations of uric acid in 100 mM sodium phosphate at pH 8.3 Data were analyzed by Eadie-Hoffstee plots, and K_m and V_{max} of each enzyme was determined.

Urate Oxidase Source	V_{max} (μ mol/min/mg)	K_m (μ M)
<i>Candida utilis</i>	10	20
<i>Arthrobacter protoformiae</i>	8	50
<i>Aspergillus flavus</i>	8	61
Hog liver	5	20

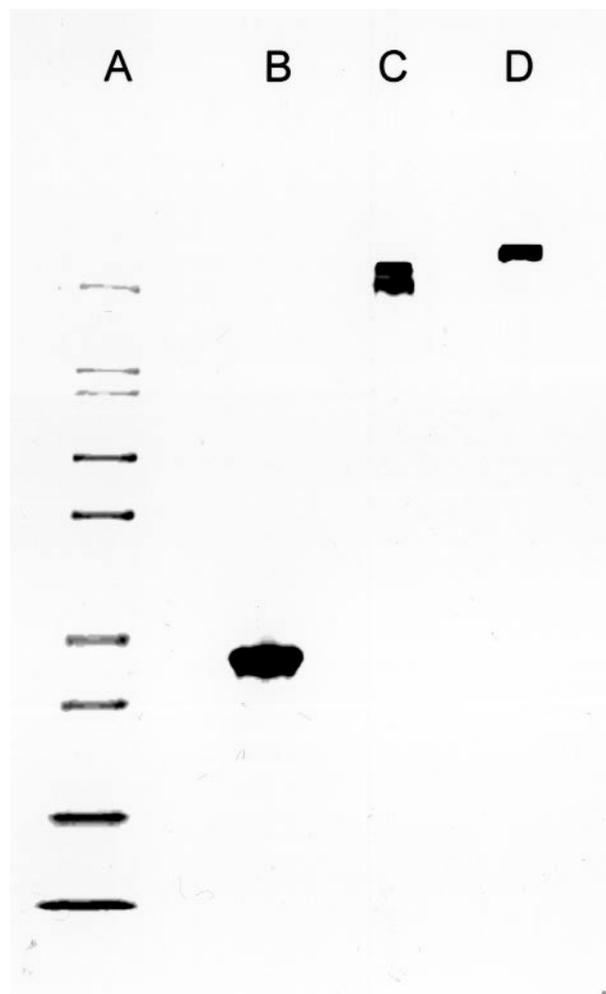


Figure 3. SDS-PAGE of uricase and PEG-uricase. Lane A: molecular weight standards (starting at top: 200, 116.3, 97.4, 66.3, 55.4, 36.5, 31, 21.5, 14.4 kDa). Lane B: recombinant urate oxidase. Lane C: enzyme formulated with PEG 5000 MW. Lane D: enzyme formulated with PEG 20,000 MW.

of the animals treated. These data indicate that nearly all (~87%) of the injected uricase-PEG 20 can be accounted for after injection. Thus it would appear that this drug is not taken up to a significant extent by cells, but instead remains in the plasma.

Mice have also been used to determine the circulating half-life of several native PEG uricase enzymes. We compared the circulating half-life of uricase-PEG 20 calculated from the combined means of male and female mice in Figure 3 with the data from Chen, *et al*²⁶ and Nishimura, *et al*²⁸ and those data are shown in Table 4.

Although the number of PEG molecules attached to the uricase protein was roughly the same in all the preparations, formulation of uricase with PEG 20,000 MW resulted in a significant increase in both the SEA and the circulating half-life of this protein in mice. Both these attributes are expected to greatly reduce the amount of protein that needs

to be administered and to reduce the frequency of administration.

Toxicology studies. Immunogenicity testing of uricase-PEG 20 in mice. To determine if PEG would also inhibit the immunogenicity of native urate oxidase, uricase-PEG 20 and uricase-PEG 5 were tested for their ability to induce an immune response, as evident by the production of anti-urate oxidase antibodies. In these experiments CD-1 mice were injected (IM) once a week (240 IU/m²) for 8 weeks and the production of antibodies determined by ELISA (Figure 5). All mice injected with uricase-PEG 5 developed an antibody response. In contrast, antibody development was unusual in mice injected with uricase-PEG 20. Both uricase-PEG 20 and uricase-PEG 5 were less immunogenic than unformulated native uricase.

Multiple dose toxicology testing of uricase-PEG 20 in mice. Uricase-PEG 20 was tested for its toxicity in mice. The mice were treated once a week (IM) for 4 weeks. The doses used in these studies were 20, 80, and 200 IU/m² (6.7, 26, 67 IU/kg). The highest dose tested represented the maximum feasible dose (limited by solubility of the drug and injection volume in the animals). In terms of mg uricase protein/kg body weight, the highest dose tested in this study is ~60–120 times the expected therapeutic dose (0.5–1.0 IU/kg) based on human studies with similar PEG-uricase^{22,23}.

All the animals (10 per sex per dose) were necropsied, the blood and urine collected and analyzed, and all tissues (47 tissues and organs) prepared for histological examination. There was no effect on the weight gain in any of the dose groups or food consumption or alterations in the behavior noted in any of the animals. There was also no change in any blood count or blood chemistry value or alteration in kidney function as noted by urinalysis in any animal. No alteration in the gross or microscopic appearance of any organ in any animal was observed. Because no adverse effect was seen in any animal at any of the doses tested, the no-effect level was defined as > 200 IU/m² (> 67 IU/kg). Note the known-effective human dose for uricase-PEG is about 0.5–1.0 IU/kg. Thus these data would predict an ample safety margin for uricase-PEG 20 in humans.

DISCUSSION

Urate oxidase (uricase, EC 1.7.3.3.) is a microbial enzyme we cloned from *C. utilis* and produced by expression in *E. coli*. The recombinant urate oxidase was formulated with PEG by the method used with PEG-uricase in humans^{22,23} and is used in the manufacture of PEG formulated asparaginase (pegasparaginase, OncasparTM) and adenosine deaminase (pegademase bovine, AdagenTM)²⁰; however, the molecular weight of the PEG used in uricase-PEG 20 is 20,000 MW instead of the 5000 MW used with PEG-uricase^{22,23}. This technology results in the covalent attachment of PEG 20,000 MW to the primary amines of urate oxidase using a succinimidyl succinimide linker. The PEG

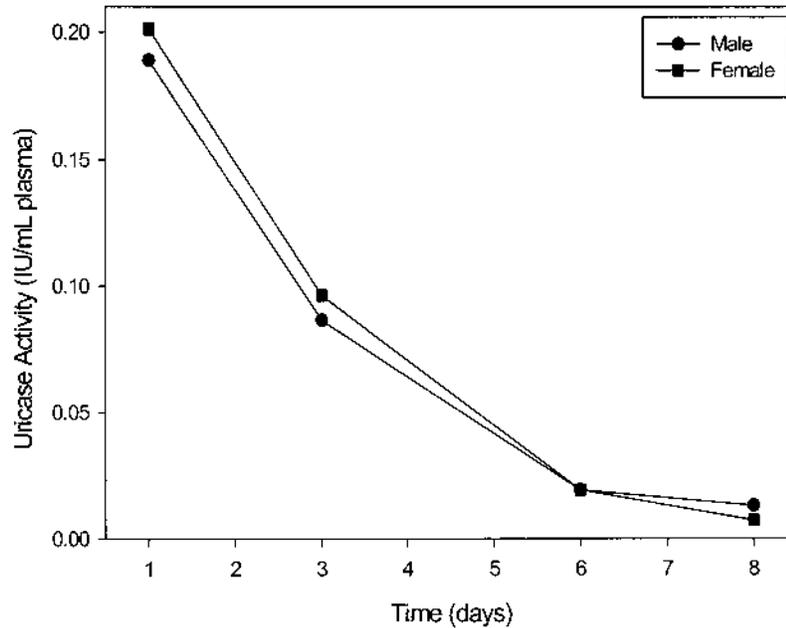


Figure 4. Pharmacokinetics of a single injection of uricase-PEG 20 in mice. CD-1 mice were given a single injection of uricase-PEG 20 and 0.04 ml plasma samples were collected at the indicated times and assayed for uricase enzyme activity. The doses tested included 20, 80, and 200 IU/m². Data shown are from animals receiving 20 IU/m²; data from other groups of animals had similar pharmacokinetics (data not shown). The means of the animals (4 mice per sex) were calculated and the results are shown.

formulated uricase-PEG 20 is provided as an isotonic sodium phosphate buffered sterile solution.

Uricase-PEG 20 has shown safety in experimental animals and has enhanced pharmacokinetic properties (longer circulating half-life) compared with native urate oxidase and other urate oxidase-PEG formulations.

Because humans do not express urate oxidase, except for a 10 amino acid fragment of the N-terminus², any urate oxidase would be expected to be seen by the immune system as a foreign protein. Thus it would seem desirable to treat patients with as little of this protein as possible to achieve the desired therapeutic end. Taken together, our data would indicate that recombinant urate oxidase isolated from *C.*

utilis has the best combination of biochemical features, including the highest affinity and the greatest specific activity at human physiological pH. In addition, urate oxidase is easy to produce as a soluble protein in *E. coli* and a simple purification procedure is available. In addition, *E. coli* do not introduce highly antigenic epitopes on recombinant proteins, unlike the yeast *S. cerevisiae* expression systems, which can hyperglycosylate recombinant proteins with a nonhuman stereo chemistry, as used by Sanofi for production of recombinant uricase (rasburicase) in Europe. Further, the *C. utilis* urate oxidase is very stable when stored at 4°C for at least one year, and can also be frozen and thawed up to 4 times with no measurable loss in enzyme activity. Thus, as any urate oxidase would be viewed as a foreign protein, the choice can be made based on the combination of most desirable features both from a biochemical perspective and from a manufacturing perspective. For these reasons we chose to utilize urate oxidase from *C. utilis* produced in *E. coli*.

Uricase from *C. utilis* has also been formulated with PEG 5000 MW using a cyanuric chloride linker^{28,29}. Similar studies were performed with uricase from *C. utilis* and formulated with various PEG, again using the cyanuric chloride linker³⁰. Decreased immunogenicity and prolonged circulating half-life in mice were noted. However, the cyanuric chloride linker is not used due to release of cyanide.

The results from immunogenicity testing confirm that formulation of urate oxidase with PEG appears to signifi-

Table 4. Comparison of half-life of various native and PEG-uricase in mice. Half-life of uricase-PEG 20 in mice was compared with pharmacokinetics of various preparations of uricase in mice reported by Chen, *et al*²⁶ and Nishimura, *et al*²⁸. Recombinant uricase-PEG 20 has not only a higher specific enzyme activity (SEA) but also a much longer circulating half-life when injected into mice.

Reference	Enzyme	Formulation	SEA, IU/mg	Half-life
Uricase-PEG 20	<i>C. utilis</i>	PEG 20,000 MW	8.5	3 days
Chen	<i>C. utilis</i>	Native	3.3	3 h
Chen	<i>C. utilis</i>	PEG 5000 MW	0.5	8 h
Chen	Hog liver	Native	2.0	3 h
Chen	Hog liver	PEG 5000 MW	0.2	6 h
Nishimura	<i>C. utilis</i>	Native	Not reported	~1 h
Nishimura	<i>C. utilis</i>	PEG 5000 MW	Not reported	~7 h

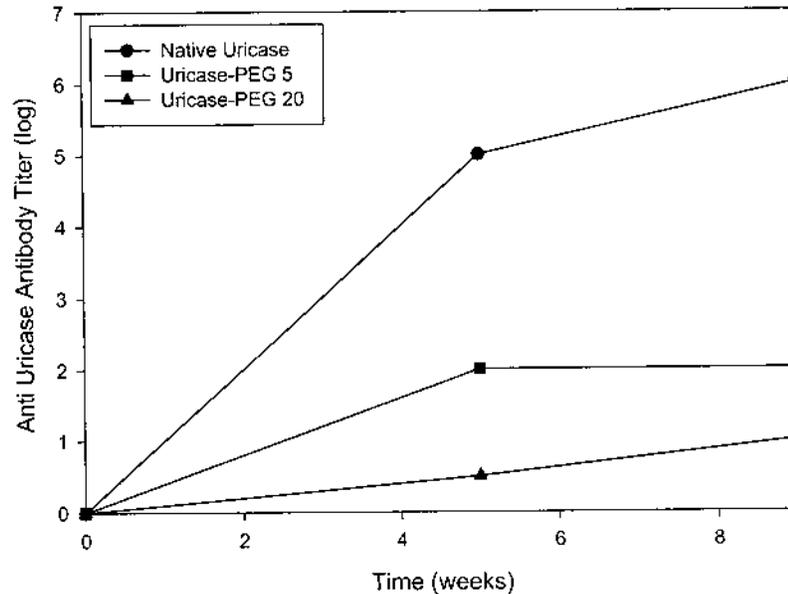


Figure 5. Immunogenicity of native urate oxidase, uricase-PEG 20, and uricase-PEG 5 in mice. CD-1 mice (4 females in each group) were injected IM once a week for 8 weeks with 80 IU/m² of either native uricase (positive control), uricase formulated with PEG 5000 MW (uricase-PEG 5), or uricase formulated with PEG 20,000 MW (uricase-PEG 20). Plasma was collected before the first injection and 7 days after the 4th and 8th injections. The titer of anti-uricase antibody was determined by ELISA and the results shown represent the means of the animals in each group.

cantly decrease the immunogenicity of the protein. Moreover, PEG 20,000 MW results in a formulation of PEG-uricase (uricase-PEG 20) that is even less immunogenic than the uricase used previously in humans (uricase-PEG 5)^{22,23,27}. Uricase from *C. utilis* or *Bacillus fastidiosus* linked to PEG 10,000 MW has been found to be less immunogenic than uricase-PEG 5 compared to native uricase in mice^{31,32}. Further, recombinant uricase from *Aspergillus flavus* produced in *S. cerevisiae* (rasburicase) resulted in antibodies to the enzyme in 17/121 (14%) patients^{17,18}.

Also, formulation of uricase with PEG 20,000 MW resulted in a significant increase in both the specific enzyme activity and the circulating half-life of this protein in mice (Figures 4 and 5). Both these attributes are expected to greatly reduce the amount of protein that needs to be administered and to reduce the frequency of administration. A pig-baboon chimeric uricase has been produced in *E. coli* coupled to PEG; this uricase is sparingly soluble at pH 7.4³³.

Thus, uricase-PEG 20, compared to uricase-PEG 5 and native uricase, is less immunogenic, has a higher SEA, and circulates longer.

Uricase-PEG 20 was tested for toxicity in mice. No adverse effects were seen in any animal at any dose tested. Therefore the no-effect level dose is > 200 IU/m² (> 67 IU/kg). In terms of mg uricase protein/kg body weight, the highest dose tested in this study is ~60–120 times the expected therapeutic dose (0.5–1.0 IU/kg) based on

previous human studies with similar PEG-uricase^{22,23}. These data would indicate uricase-PEG 20 may be useful in the treatment of humans, in place of allopurinol, in the treatment of gout and other hyperuricemic states — patients with gout and renal insufficiency who are allergic to allopurinol and in whom allopurinol desensitization has failed; rapid and more effective control of malignancy associated hyperuricemia resulting from tumor lysis syndrome; and suboptimal response to allopurinol in cyclosporine treated transplant recipients with renal failure and severe hyperuricemia and gout³⁵.

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