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GLUCOSE OXIDASE AS A PHARMACEUTICAL ANTIOXIDANT

Thomas F. Berg[1] and Paul J. Niebergall[2]

Department of Pharmaceutical Sciences
Medical University of South Carolina
171 Ashley Avenue
Charleston, SC 29425-2303

ABSTRACT

The effect of five antioxidant systems on the rate of oxidation of isoproterenol formulations was studied. In addition to a control, the antioxidants were studied alone and in all possible combinations of two. The systems tested were glucose oxidase, nitrogen, sodium bisulfite, acetylcysteine and thiourea. Glucose oxidase and its combination with nitrogen flushing or thiourea were found to be effective. Acetylcysteine appeared to inactivate glucose oxidase. The combination of sodium bisulfite and thiourea was also effective although nitrogen or thiourea alone were not effective.

[1] Present address: University of Arkansas for Medical Sciences
College of Pharmacy
Slot 522
4301 W. Markham
Little Rock, AR 72205

[2] To whom correspondence should be addressed

INTRODUCTION

Although a reasonable number of drugs are subject to oxidative degradation in aqueous solution, there are a limited number of antioxidants that can be used. The agents used today have a number of limitations. For example, the sulfites, the most common antioxidants in aqueous solution have pH stability and usefulness ranges. Sodium bisulfite is most effective between pH 1 - 5, while sodium sulfite is more stable and effective between pH 7 - 10. The sulfites, however, often react with the drugs they are meant to protect, frequently resulting in loss of potency(1,2,3). The safety of the sulfites is under attack. In 1986 the FDA revoked the GRAS status of sulfiting agents for use on fruits and vegetables intended to be served raw. It also required that the presence of a detectable amount of sulfite (10 ppm) in a food, in which the sulfite does not have a technical or functional effect, must be declared on the label(4,5).

Removal of oxygen, while generally not complete, can increase the stability of some drugs toward oxidation. Glucose oxidase, an enzyme known since 1926 which is used in the food and beverage industries(6,7,8,9) reacts with glucose while removing dissolved oxygen in aqueous systems. It was felt that its utility in pharmaceutical systems warranted study.

MATERIALS AND METHODS

All chemicals used were A.C.S reagent grade, with the exception of glucose oxidase which was an industrial grade supplied by Boehringer Mannheim Biochemicals. All materials were used as supplied.

Removal of Oxygen from Solution. A stock buffer solution at pH 8.5 was made by dissolving 25.99 g boric acid and 29.84 g potassium chloride in distilled water. The solution was adjusted to pH 8.5 with

sodium hydroxide and brought to volume. A sufficient amount of dextrose was dissolved in 200 ml of this buffer to give a final concentration of 75 mg/ml. Two hundred ml of the dextrose solution were placed in a 250 ml erlenmeyer flask. The flask was placed on a magnetic stirrer. An Orion Model 97-08 oxygen electrode was inserted and allowed to equilibrate. The oxygen content in ppm was measured using an Orion pH meter Model 811. One ml of a solution containing 75 units/ml of glucose oxidase were added and the oxygen content was measured as a function of time.

Oxidation of Isoproterenol. All solutions contained isoproterenol hydrochloride 0.2 mg/ml in distilled water at pH 6. The antioxidants, their amounts and combinations are given in TABLE 1. The solutions containing nitrogen were made by bubbling nitrogen through distilled water, preparing the solutions, purging the solutions before filling and filled with a nitrogen head. The solutions were placed in 5 ml Type 1 amber glass ampuls(Owens-Illinois) with a Cozzoli F400X ampul filler and sealed with a Cozzoli HS1 ampul sealer. All stock solutions were prepared in light resistant Class A volumetric flasks. The sealed ampuls were placed in an incubator at 45° C. Samples were removed at intervals and assayed for isoproterenol content by HPLC. Three injections were made per sample and the concentration was determined from their mean.

HPLC Assay. A modification of the assay described by Nayak(10) was used to follow the degradation of isoproterenol. The assay was carried out using a Waters Model 510 pump, waters Model 481 Variable Wavelength Detector, Shimadzu C-R3A Integrator/Recorder, Rheodyne injector with a 20 μL loop. The column was a 10 cm x 4.6 cm Rainin Microsorb 5 μ. The assay was isocratic with a mobile phase that consisted of 1% acetic acid and 2.5% methanol. The flow rate was 1 ml/min and the detector wavelength was 273 nm. The run time was 20 minutes. Sample chromatograms showed baseline separation of

Table I. First Order Rate Constants and Percent Remaining after 81 Days at 45°C

Antioxidant	Concentration	k x 10^3 ($days^{-1}$)	Percent Remaining
None	---------	16.50	29.2
Nitrogen	saturated	2.46	80.1[1]
Sodium Bisulfite	0.5 mg/ml	2.71	73.0
Acetylcysteine	5.0 mg/ml	0.21	76.6
Thiourea	1.0 mg/ml	15.92	28.4
Glucose Oxidase[2]	250 u/ml	0	99.4
Nitrogen Sodium Bisulfite	saturated 0.5 mg/ml	2.71	70.6
Nitrogen Acetylcysteine	saturated 5.0 mg/ml	3.08	77.3
Nitrogen Thiourea	saturated 1.0 mg/ml	1.55	88.3
Nitrogen Glucose Oxidase[2]	saturated 250 u/ml	0	98.8
Sodium Bisulfite Acetylcysteine	0.5 mg/ml 5.0 mg/ml	4.69	68.0
Sodium Bisulfite Thiourea	0.5 mg/ml 1.0 mg/ml	0	98.8
Sodium Bisulfite Glucose Oxidase[2]	0.5 mg/ml 250 u/ml	1.50	88.6
Acetylcysteine Thiourea	5.0 mg/ml 1.0 mg/ml	3.54	75.3
Acetylcysteine Glucose Oxidase[2]	5.0 mg/ml 250 u/ml	4.43	75.5
Thiourea Glucose Oxidase	1.0 mg/ml 200 u/ml	0	96.1

[1] Extrapolated from 67 to 81 days using the slope and intercept obtained from nonlinear regression.

[2] All solutions with glucose oxidase had 50 mg/ml of glucose.

isoproterenol from the other components of the solutions. The coefficient of variation for six replicates of a standard solution was 0.7%. The response was linear over the range of interest with an R^2 of 99.91%.

RESULTS

FIGURE 1 shows the rate at which oxygen is removed from a solution containing 75 mg/ml of dextrose and 0.375 units/ml of glucose oxidase. The oxygen content dropped from 9 ppm to about 0.8 ppm in approximately 20 minutes, and levelled off at about 0.3 ppm within 50 minutes. Since this was a preliminary study on the possible effectiveness of glucose oxidase as an antioxidant in a pharmaceutical system, no further attempts were made to optimize the amounts of the enzyme or dextrose. These studies are now underway.

FIGURE 2 shows data for the loss of isoproterenol as a function of time for the control and the single antioxidant systems. The figure indicates that glucose oxidase alone appears to be superior to the other single antioxidant systems, while thiourea appeared to have no stabilizing effect.

The antioxidant concentrations, first order rate constants determined using the nonlinear regression analysis program MINSQ(MicroMath, Inc.) and the percent of isoproterenol remaining after 81 days at 45°C are given in TABLE I. The rate constants for the glucose oxidase, glucose oxidase-nitrogen, and glucose oxidase-thiourea systems were not significantly different from zero. The percent of isoproterenol remaining after 81 days ranged from 96.1 to 99.4 percent for these systems. These data indicate that glucose oxidase warrants further investigation as an antioxidant in pharmaceutical systems. Acetylcysteine appeared to have a deleterious effect on the glucose oxidase with only 75.5 percent remaining after 81 days. This is

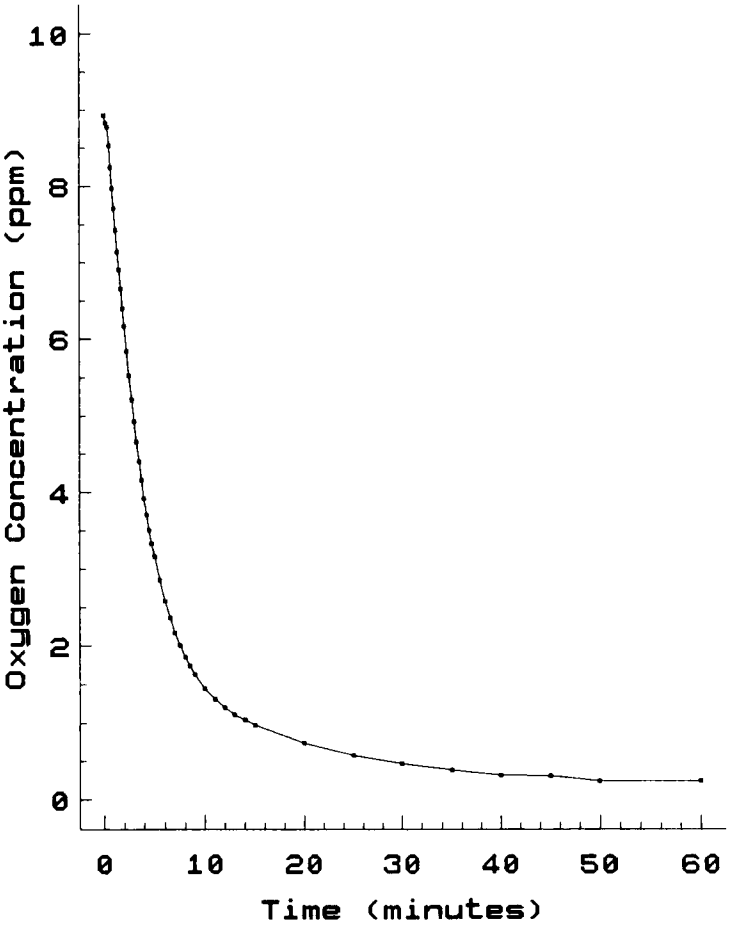

FIGURE 1 - Oxygen remaining in ppm as a function of time.

approximately the same as for the system containing only acetylcysteine(76.6% remaining).

None of the other antioxidant systems used alone appeared to be effective. The combination of sodium bisulfite and thiourea appeared to be effective with a rate constant deemed not to be significantly different from zero, and 98.8% isoproterenol remaining after 81 days. The mixture was far superior than sodium bisulfite or thiourea alone. None of the other combinations were effective.

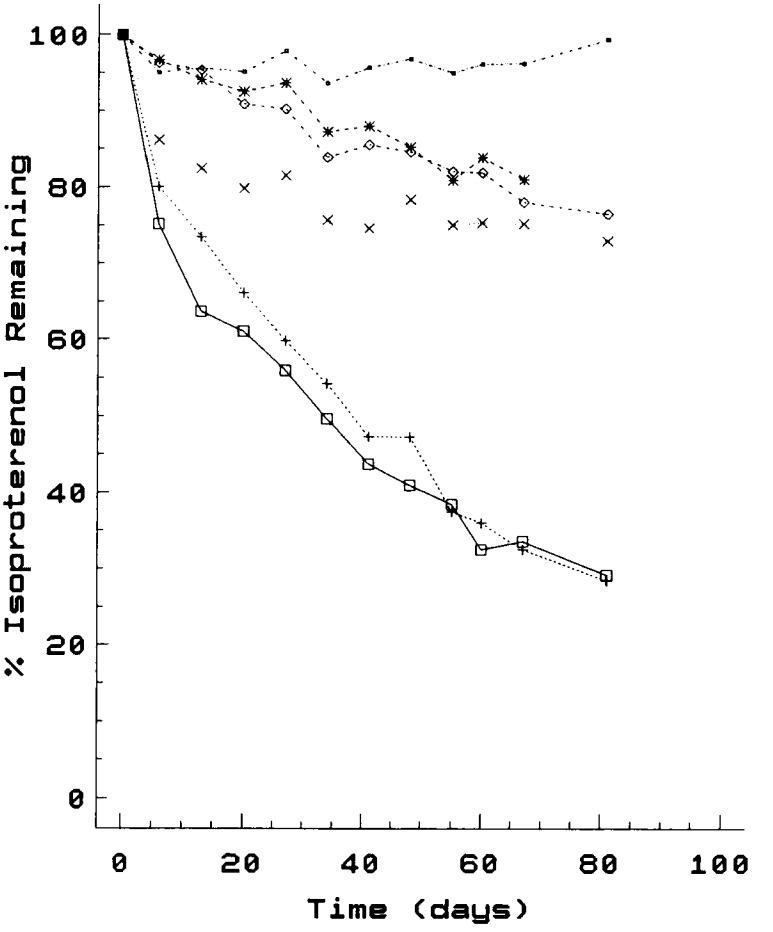

FIGURE 2 - Percent isoproterenol remaining after storage for up to 81 days at 45° C. □ control; * nitrogen flush; x sodium bisulfite; -o- acetylcysteine; + thiourea; -•- glucose oxidase.

DISCUSSION

Glucose oxidase was first described by Müller(6) in 1926 from the press juice of Aspergillus niger, and later from Penicillium glaucum. He demonstrated that the enzyme catalyzed the oxidation of glucose to gluconic acid. Coulthard and coworkers(10) succeeded in preparing a highly purified form from penicillium notatum. They named the enzyme notatin. Van Brugger and coworkers(11) independently purified the same enzyme, calling it penicillin B. Kielin and Hartree(12) confirmed that the enzyme was a flavoprotein, and that the prosthetic group was alloxazine-adenine dinucleotide(riboflavin-phosphate-phosphate-ribose-adenine). They concluded that glucose oxidase, notatin and penicillin B were the same enzyme. The Enzyme Commission designation is β-D-glucose: oxidoreductase E.C. 1.1.3.4. It is still most widely know as glucose oxidase. Bentley and Neuberger(13) demonstrated that the oxygen consumed is dissolved oxygen and not from water molecules. Most commercial grades of glucose oxidase contain at least small amounts of catalase as well.

The overall reactions involved in the removal of dissolved oxygen are:

$$2C_6H_{12}O_6 + H_2O + 2O_2 \xrightarrow[\text{oxidase}]{\text{glucose}} 2C_6H_{12}O_7 + 2H_2O_2 \qquad (1)$$

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2 \qquad (2)$$

The net reaction is:

$$2C_6H_{12}O_6 + O_2 \xrightarrow[\text{catalase}]{\text{glucose oxidase}} 2C_6H_{12}O_7 \qquad (3)$$

Glucose oxidase is specific for glucose and has been used as a component of home tests for glucose in urine. It is used in the food industry to desugar egg albumin and whole eggs prior to drying(14). It has been shown to protect unpasteurized beer from spoiling at room temperature for up to 50 days(15). Unpasteurized beer, if unprotected, will spoil in seven to twelve days at room temperature. It has been used to protect mayonnaise(16) and animal feeds(17). It is reported to be effective over the pH range of 2 - 8 and is stable for eight years at 15° C.(6).

COMMENTS AND CONCLUSIONS

1. Glucose oxidase was found to be an effective antioxidant system for isoproterenol.
2. Glucose oxidase combined with nitrogen or thiourea were also effective, but no more so than glucose oxidase alone.
3. The sodium bisulfite-glucose oxidase had more of an effect(88.6% left after 81 days) than sodium bisulfite alone(73 % left after 81 days). The combination was not as effective as glucose oxidase alone.
4. Acetylcysteine appeared to inactivate glucose oxidase.
5. The combination of sodium bisulfite and thiourea was also effective in protecting isoproterenol from oxidation.
6. Thiourea had no stabilizing effect at all.

Further studies are underway to fully characterize the conditions and possible optimization for using glucose oxidase as an oxidant. This will include determining whether glucose oxidase as currently available is pyrogenic.

REFERENCES

1. L.C. Schroeter, T. Higuchi and E.E. Schuler, J. Am. Pharm. Assoc., Sci. Ed., 47, 723(1958).

2. T. Higuchi and L.C. Schroeter, ibid, 48, 535(1959).

3. L.C. Schroeter, J. Pharm. Sci., 50, 891(1961).

4. Federal Register, 51, 25021, July 9, 1986.

5. Federal Register, 51, 25012, July 9, 1986.

6. L.A. Underkofler, in "Proceedings of the International Symposium on Enzyme Chemistry,"2, 486(1957).

7. D. Ohlmeyer, Food Tech., 11, 503(1951).

8. D. Scott, Food Tech., 12, 7(1959).

9. A. Koczot and J. Zaleski, J. Prezm. Ferment. Rolny., 20, 18(1976).

10. A.S. Nayak, A.J. Cutie, T. Jochsberger, and A.I. Kay, Drug Devel. and Ind. Pharm., 12, 589(1981).

11. R. Bentley, in "The Enzymes," Vol. 7, P.D. Boyer, H. Lardy, K. Myrbäck, eds.,Academic Press, New York, 1963, p.567.

12. H. Theorell, "Flavin-Containing Enzymes," Vol. II, Part 1, J.B. Sumner and K. Myrbäck, eds., Academic Press, New York, 1951, p.348.

13. D. Keillin, and E.F. Hartree, Biochem. J., 42, 230(1949).

14. R. Bentley and A. Neuberger, Biochem. J., 45, 584(1949).

15. D. Scott, Agricultural and Food Chemistry, 1, 727(1953).

16. D. Ohlmeyer, Food Tech., 11, 503(1951).

17. D. Scott, Food Tech., 12, 7(1959).