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Quantitation of hydroperoxides in the aqueous solutions of non-ionic surfactants using polysorbate 80 as the model surfactant

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Abstract: The application of the coupled oxidation of NADPH to peroxide quantitation in non-ionic surfactant solutions is demonstrated using polysorbate 80 as the model surfactant. The linearity, precision, accuracy, and sensitivity of the method are discussed. The method has the following advantages over the traditional iodimetric method: (1) it is not affected by the non-ionic surfactant present in the solution; (2) it is reactive to less reactive hydroperoxides; (3) it is not light sensitive; and (4) it is carried out at near physiological pH in aqueous solutions. The method was employed to monitor the peroxide concentration of polysorbate 80 solutions stored under three different conditions. The effects of light and heat on peroxide concentration are more pronounced in more dilute solutions (0.1 and 1%). The peroxide concentration determined in the freshly prepared polysorbate 80 solution can be converted to the peroxide number of the raw material, which is not available from the supplier of polysorbate 80.

Keywords: *Hydroperoxides; NADPH; non-ionic surfactant; peroxide concentration; polysorbate 80.*

Introduction

Non-ionic surfactants are widely used ingredients in pharmaceutical and cosmetic formulations. However, they are not free of physicochemical concerns. Some non-ionic surfactants are susceptible to hydrolysis [1], and others to autoxidation [2–4]. A few non-ionic surfactants, such as nonoxynol and octoxynol, may themselves be stable but these raw materials can contain oxidizing impurities. Formulation problems related to non-ionic surfactants have been reported [5–7], supporting the need for careful selection.

The peroxide concentration in the aqueous solution of non-ionic surfactants is a subject of interest in this laboratory since peroxide concentration is a chemical indicator of oxidative instability and oxidatively unstable excipients are more likely to cause formulation problems than those oxidatively stable. Initially, the widely used spectrophotometric iodide method [8] was employed as the assay method for peroxides. The method was sensitive but it was limited for a number of reasons. First, non-ionic surfactants were found to interfere with the colour development of the liberated iodine. The yellow colour of the triiodide complex was quickly quenched after the addition of a small quantity of a non-ionic surfactant such as Pluronic® P85 or nonoxynol. Similar interference was also noticed by other researchers [9, 10]. Non-ionic surfactants were reported to interact with the liberated iodine and thereby a portion of iodine was unavailable for titration. Another drawback of the iodimetric method was its low reactivity toward less reactive peroxides [8]. Since hydroperoxides of this type were likely to be produced by non-ionic surfactants, the method was concluded to be inadequate for our interest.

To overcome the problems associated with the iodimetric method, the coupled oxidation of NADPH was considered. The system requires the presence of two enzymes, glutathione peroxidase and reductase, for its application to peroxide quantitation, and is usually known for its biochemical applications in the analysis of enzyme activities or metabolites in biological samples [11]. The method was evaluated for its feasibility in quantitating peroxides in the presence of non-ionic surfactants using polysorbate 80 as the model surfactant. Various aspects of the method (linearity, precision, accuracy, and sensitivity) were studied. Finally, the peroxide concen-

trations of polysorbate 80 solutions stored under three different conditions were followed.

Experimental

Chemicals and enzymes

All chemicals were USP or AR grade. Sodium azide (99%) and reduced glutathione (98%) were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA). Glutathione peroxidase (200 units, from bovine erythrocytes), glutathione reductase (2500 units, type III from yeast), and β-nicotinamide adenine dinucleotide phosphate reduced form (β-NADPH, tetrasodium salt, type III, 98%) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Hydrogen peroxide (30%) was 'Baker Analyzed' Reagent purchased from J.T. Baker (Phillipsburg, NJ, USA). The hydrogen peroxide concentration was determined to be 34.4% (w/v) by titrating with potassium permanganate. Polysorbate 80 (Tween® 80, NF, Lot No. 20621L) was a gift from ICI Americas Inc. (Wilmington, DE, USA). It had been stored in the dark since being received.

Stock solutions

Stock solutions were prepared in double distilled and deionized water. They were prepared and handled according to standard laboratory procedures unless otherwise stated.

Glutathione (GSH) peroxidase stock solution. This was prepared by adding 10 ml of water to the vial containing approximately 200 units of the lyophilized enzyme powder. The solution was shaken, dispensed into Teflon-lined screw-capped Wisp vials (5 ml), and frozen immediately. The frozen solution was defrosted before use by standing at room temperature for 30–60 min.

Glutathione (GSH) reductase stock solution. This was prepared by transferring 50 μl of the 2500-unit enzyme solution to a 10 ml volumetric flask. The solution was brought to the mark with water, mixed well, dispensed into Teflon-lined screw-capped Wisp vials (5 ml), and frozen immediately. The frozen solution was defrosted before use by standing at room temperature for 30–60 min.

GSH solution (0.038 M). This was prepared fresh in degassed water. The container was flushed with nitrogen and capped tightly before use.

β-NADPH solution. This was prepared fresh by dissolving 6.0 mg of β-NADPH per millilitre of water.

Hydrogen peroxide standards. These were prepared fresh by diluting the 30% hydrogen peroxide reagent serially.

Sample preparation

Typical sample compositions are shown in Table 1. Samples were prepared by accurately transferring stock solutions of the required reagents/enzymes and the testing material into a Teflon-lined screw capped Wisp vial (5 ml).

Table 1
Typical sample compositions of the coupled oxidation of NADPH

Reagent	10% Polysorbate 80 sample (ml)	Hydrogen peroxide standard (ml)	Control (ml)	Final conc.
0.5 M Phosphate buffer, pH 7.87	0.2	0.2	0.2	0.05 M
0.005 M Disodium edetate	0.2	0.2	0.2	5×10^{-4} M
0.052 M Sodium azide	0.2	0.2	0.2	5.2×10^{-3} M
~20 U ml^{-1} glutathione peroxidase	0.1	0.1	0.1	~1 U ml^{-1}
~12.5 U ml^{-1} glutathione reductase	0.2	0.2	0.2	~1.25 U ml^{-1}
~6 mg ml^{-1} NADPH	0.1	0.1	0.1	3.5×10^{-4} M
0.038 M GSH	0.2	0.2	0.2	3.8×10^{-3} M
Water*	0.7	0.6	0.8	—
H_2O_2 Standard (8.088×10^{-4} M)	—	0.2‡	—	8.088×10^{-5} M
10% Polysorbate 80 solution	0.1†	—	—	0.5%
Total volume	2.0	2.0	2.0	

*The subtotal volume of the first seven reagents is 1.2 ml, and that of water and a peroxide sample is 0.8 ml.
†The volume may vary up to 0.8 ml for 0.1 and 1% polysorbate 80 solutions.
‡The volume may vary up to 0.8 ml.

The 0.038 M GSH stock solution was added last. Immediately after the addition of the GSH stock solution, the vial was flushed with nitrogen, capped, and vortexed.

Absorbance measurement

The absorbance at 340 nm was taken between 50 and 70 min after the start of the reaction. The cuvette was flushed with nitrogen and capped immediately to minimize the oxidation of GSH by free oxygen.

Time course study

The samples were directly prepared in the cuvette. After the cuvette was flushed with nitrogen and capped, the absorbance at 340 nm was monitored as a function of time.

Storage conditions of polysorbate 80 solutions

Polysorbate 80 aqueous solutions (0.1, 1 and 10%) were stored under three different conditions: ambient (25°C) dark, ambient (25°C) light, and 45°C dark. The light intensity to which the ambient light samples were exposed was approximately 200–230 foot-candles. The container used for storage was a 1-l screw-capped glass bottle. Approximately 300 ml of the polysorbate 80 solution was placed in each bottle. At each sampling time, an aliquot was withdrawn from each solution for assay and the solutions were swirled, capped, and returned to their storage areas.

Results and Discussion

Reaction principle

The principle of the coupled oxidation of NADPH in peroxide quantitation is described below in two equations:

$$2GSH + ROOH \xrightarrow{\text{Glutathione peroxidase}} ROH + GSSG + H_2O, \quad (1)$$

where R may be H or an organic residue; and

$$GSSG + NADPH + H^+ \xrightarrow{\text{Glutathione reductase}} 2GSH + NADP^+. \quad (2)$$

Peroxides oxidize the reduced glutathione (GSH) and convert it to the oxidized form (GSSG) through catalysis by glutathione peroxidase. The amount of GSSG produced can be measured by following the disappearance of NADPH in its coupled oxidation by GSSG. The pronounced lack of selectivity of glutathione peroxidase toward acceptor substrates is key to the success of the method in picking up different kinds of hydroperoxides. Reportedly, the substrates range in complexity from hydrogen peroxide to nucleic acid and steroid hydroperoxides [8].

Response profile of the coupled oxidation of NADPH

A time course study was conducted to learn the response profile of the coupled oxidation of NADPH to different oxidative species such as free oxygen, highly reactive hydrogen peroxide, and peroxides produced by degraded non-ionic surfactants. Figure 1 shows the decline of the absorbance at 340 nm as a function of time in the sample containing hydrogen peroxide or aged polysorbate 80. The profile of the corresponding control is also included in the figure. Figure 2 is the differential plot of Fig. 1, showing the change in the slope of each response profile as a function of time.

As shown in Figs 1 and 2, the absorbance decline in the control is gradual and slow; it stabilizes at approximately 10 min after the start of the reaction with a slope of approximately 0.0006 units per minute. The decrease in absorbance in the control is attributed to the disappearance of NADPH due to the non-peroxide oxidative species such as the free oxygen dissolved in the solution. The response profile of the hydrogen peroxide sample is biphasic, showing a rapid initial decline, followed by a gradual and slow change whose rate is similar to that of the control. The second phase begins about 5 min after the start of the reaction, indicating that the coupled oxidation initiated by hydrogen peroxide is completed by 5 min and the absorbance decrease post 5 min is due to non-peroxide oxidative species.

The response profile of the polysorbate 80 solution is triphasic, showing a rapid initial decline, followed by a slower decrease, and then the terminal phase whose slope is similar to that of the control. The terminal phase does

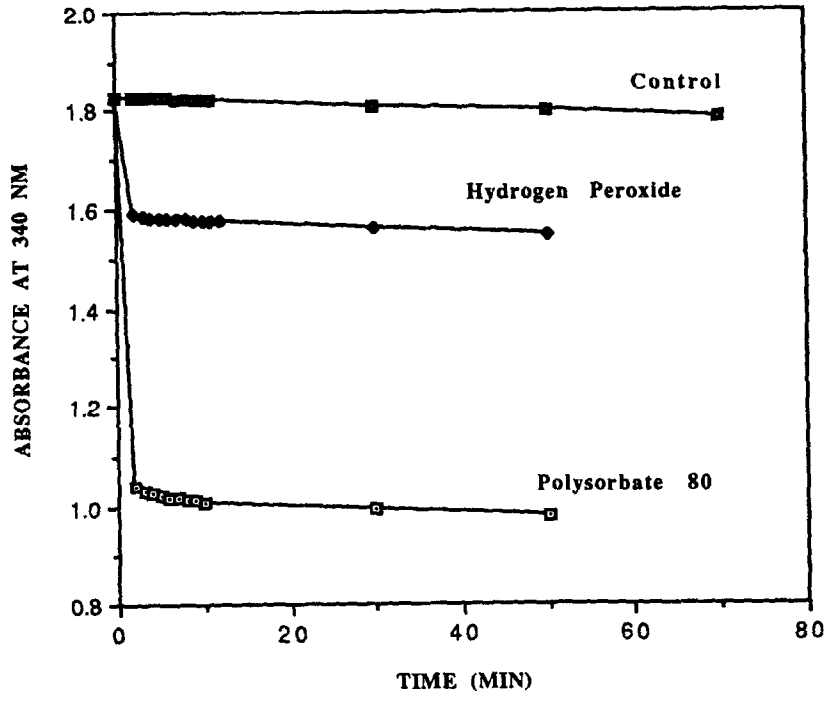

Figure 1
The response profile of the coupled oxidation of NADPH.

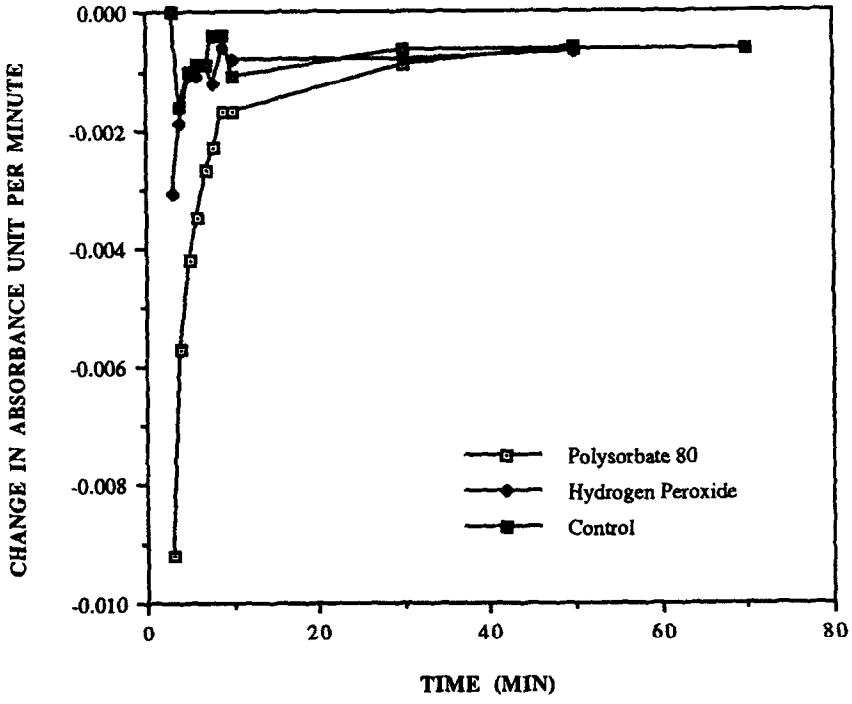

Figure 2
The differential time course plot of the coupled oxidation of NADPH.

not begin until 30 min after the start of the reaction.

The results of the time course study suggest that the coupled oxidation of NADPH initiated by non-peroxide oxidative species should not be neglected. However, since it reacts at the same rate in all samples, the quantity of NADPH consumed by this source can be estimated using the control. Another important implication suggested by the results is that some peroxides present in the aged polysorbate 80 solution are less reactive, and it may take 30 min to complete the coupled oxidation initiated by them. The highly reactive peroxides such as hydrogen peroxide are completely reacted within 5 min.

In light of the results of the time course study, the absorbance of each sample was read between 50 and 70 min after the start of the reaction. The decision to read between 50 and 70 min was made based on convenience of handling and the time (at least 30 min) required to complete the coupled oxidation initiated by less reactive peroxides. The error introduced by the possible difference in measuring time (maximum 20 min) is approximately 0.001–0.002 absorbance unit, which accounts for less than 1% of error.

Linearity

The difference in the absorbance (ΔA_{340nm}) between the control and the peroxide containing sample accounts for the NADPH consumed by the coupled oxidation initiated by peroxides. Using hydrogen peroxide as the standard, a linear relationship ($r = 0.9999$) was found between ΔA_{340nm} and H_2O_2 concentration in the concentration range of 2×10^{-5} to 2.4×10^{-4} M (Fig. 3). The slope of the straight line is $6.24 \times 10^3\ M^{-1}\ cm^{-1}$, which is consistent with the literature value [8], and the y-intercept is -0.00323. Because the y-intercept is sufficiently close to the origin, a single standard may be used for the assay in the concentration range established for linearity.

The standard curve loses its linearity and begins to alter when the hydrogen peroxide concentration is above 2.5×10^{-4} M or below 2×10^{-5} M. The detection limit of the method is $6–7 \times 10^{-6}$ M (approximately equivalent to 0.25 ppm).

One advantage of the method is that its standard curve is independent of peroxide species assayed. Frew *et al.* [8] reported that the standard curves generated from five different peroxides were congruent. This advantage is particularly important in the effort to quan-

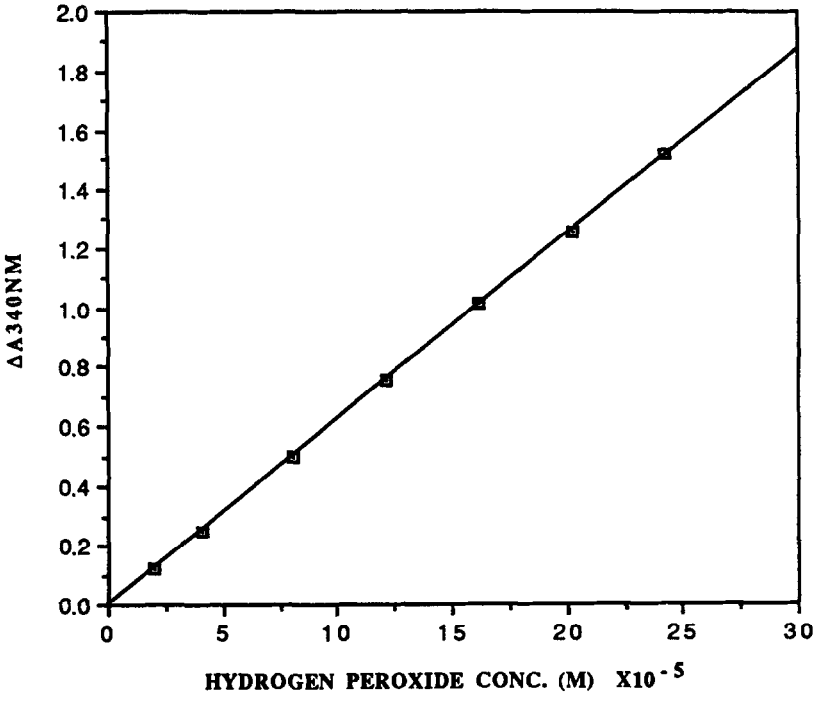

Figure 3
Standard curve of the coupled oxidation of NADPH.

titate peroxides for non-ionic surfactants since the nature of the peroxide is unknown.

Precision and accuracy

The precision and accuracy of the method in quantitating peroxide in the presence of a non-ionic surfactant was studied by spiking a known amount of hydrogen peroxide to the samples containing 0.5% (w/v) polysorbate 80. As shown in Table 2, the average recovery at the spiked concentration of 4.044×10^{-5} M is 101.3%, and the reproducibility is within ±2.3% relative standard deviation (RSD). The method is concluded to be reasonably precise and accurate at the concentration of polysorbate 80 up to 0.5%.

Peroxide content in polysorbate 80 solutions

The validated NADPH method was employed to determine the peroxide concentration in 0.1, 1 and 10% of polysorbate 80 aqueous solutions stored under three different conditions. The study lasted for only 1 month because an indication of molding began to show in some of the samples after 4 weeks.

Table 3 summarized the results of the study. As shown in the table, the method successfully monitors the peroxide level in the samples and the results obtained are accurate and consistent. The detection of peroxides through the study suggests a continuous formation of peroxides. The detrimental effects of light and heat on the oxidative stability of polysorbate 80 can be seen clearly in 0.1 and 1% solutions since a higher peroxide concentration was found in the samples stored either at a higher temperature (45°C) or with light exposure.

As to the peroxide concentration profile, a gradual increase in peroxide concentration was found in the solutions stored at 45°C in darkness and at ambient temperature with light exposure. The ambient dark samples yielded a different profile which increased first then decreased. This increase–decrease behaviour is believed to be due to the further degradation of perioxides which becomes more pronounced at a lower temperature in the dark.

Consistent with what is reported in the literature, the dilute polysorbate 80 solution appears to be more susceptible to oxidation. A comparison of the 45°C data of week 4 with that of week 1 reveals that the peroxide concentration increased 9-fold in 0.1% polysorbate 80 solution while only 4.5-fold and 1.5-fold increases were found in the 1 and 10% solutions, respectively, during the same period.

The peroxide concentration obtained from the freshly prepared polysorbate 80 solution can be used to calculate the peroxide number (PN) of polysorbate 80 raw material. A value of 48.4 meq kg^{-1} (i.e. PN = 48.4) is obtained using the zero time peroxide concentration shown in Table 3 for 10% polysorbate 80 solution. The value, PN = 48.4, indicates that the raw material received is contaminated with a significant amount of peroxide(s), and may not be suitable for the formulation of sensitive products.

The peroxide number is an important piece of information for materials known to be

Table 2
Precision and accuracy of the coupled oxidation of NADPH method for the quantitation of peroxides in the aqueous solutions of polysorbate 80

H_2O_2 Spiked (M) × 10^5	H_2O_2 Calc'd (M) × 10^5	Recovery (%)
4.044	4.003	99.0
4.044	4.044	100.0
4.044	4.225	104.5
4.044	4.049	100.1
4.044	4.171	103.1
Mean	4.098	101.3
SD	0.095	2.3
RSD	2.3	2.3

Table 3
Peroxide concentrations (mEq l^{-1}) in the aqueous solutions of polysorbate 80 in three storage conditions

	10% Solution			1% Solution			0.1% Solution		
Time (weeks)	25°C Light	25°C Dark	45°C Dark	25°C Light	25°C Dark	45°C Dark	25°C Light	25°C Dark	45°C Dark
0	4.84	4.84	4.84	0.50	0.50	0.50	NQ*	NQ*	NQ*
1	5.47	5.11	5.44	0.86	0.56	1.11	NQ*	NQ*	0.11
2	5.38	6.02	6.34	0.68	1.54	2.38	NQ*	0.17	0.36
3	6.56	5.62	7.07	2.30	0.62	3.37	0.34	NQ*	0.73
4	7.32	5.94	8.16	3.25	0.66	4.96	0.65	NQ*	0.96

*Detectable but not quantifiable.

oxidatively unstable. Typical examples are fats and oils; their degree of deterioration is often reflected by this number, and there is a standard method published by the American Oil Chemists' Society for the determination of the number in fats and oils [12]. For polysorbate 80, despite its popularity and well-known oxidatively unstable nature, the information regarding the peroxide number or its determination is not available from the supplier and little can be found in the literature. Research reports can be found on the hydrolysis of polysorbate 80 [1] or autoxidation of other members of the polysorbate family such as polysorbate 20 [4] but not on the autoxidation of polysorbate 80. This lack of information is due, perhaps, to the difficult nature of the task. In our laboratory, we had tried to titrate the peroxides in the polysorbate 80 raw material using the traditional iodine method but failed as no end point could be reached in the titration. The yellow colour developed again and again after each addition of the titrant.

The failure of the iodimetric titration for the raw material increases the importance of the coupled oxidation of NADPH in its application to non-ionic surfactants. It has proven to be a useful tool not only in studying the aqueous oxidative stability of non-ionic surfactants but also in evaluating the quality of the raw materials of the surfactants.

References

[1] T.R. Bates, C.H. Nightingale and E. Dixon, *J. Pharm. Sci.* **25**, 470–477 (1973).
[2] M. Donbrow, in *Nonionic Surfactants Physical Chemistry* (M.J. Schick, Ed.), pp. 1011–1072. Marcel Dekker, New York (1987).
[3] M.M. Rieger, *Cosmetics and Perfumery* **90**, 12–16 (1975).
[4] M. Donbrow, E. Azaz and A. Pillersdorf, *J. Pharm. Sci.* **67**, 1676–1681 (1978).
[5] A.G. Mitchell and L.S.C. Wan, *J. Pharm. Sci.* **54**, 699–704 (1965).
[6] S. Hayashi and Y. Nishii, *Vitamins* **43**, 269–273 (1971).
[7] J.W. McGinity, J.A. Hill and A.L. La Via, *J. Pharm. Sci.* **64**, 356–357 (1975).
[8] J.E. Frew, P. Jones and G. Scholes, *Analytica Chim. Acta* **155**, 139–150 (1983).
[9] E. Azaz, M. Donbrow and R. Hamburger, *Analyst* **98**, 663–672 (1973).
[10] W.B. Hugo and J.M. Newton, *J. Pharm. Pharmacol.* **15**, 731–741 (1963).
[11] D.E. Paglia and W.N. Valentine, *J. Lab. Clin. Med.* **70**, 158–169 (1967).
[12] W.E. Link (Ed.), *Official and Tentative Methods of the American Oil Chemists' Society*, 3rd edn. AOCS, Champaign, IL (1975).

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