

Sulfite Supported Lipid Peroxidation in Propofol Emulsions

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Background: Sodium metabisulfite is added to a commercial propofol emulsion as an antimicrobial agent. The sulfite ion (SO_3^{2-}) is capable of undergoing a number of reactions, including autooxidation and the promotion of lipid peroxidation. This study evaluated sulfite reactivity in propofol emulsions by determining thiobarbituric acid reacting substances (TBARS), sulfite depletion, and emulsion pH in emulsions containing sulfite or EDTA.

Methods: Commercial EDTA and sulfite propofol emulsions were compared, and 10% soybean oil emulsion containing various additives were evaluated for TBARS, sulfite, and pH. TBARS were analyzed with a modified thiobarbituric acid method. Sulfite was analyzed by the reaction of sulfite with 5,5'-dithiobis(2-nitrobenzoic acid). pH was measured by glass electrode methodology.

Results: Thiobarbituric acid reacting substances were detectable in commercial sulfite propofol emulsions in concentrations ranging from 0.02 to 0.22 $\mu\text{g}/\text{ml}$ based on malondialdehyde. No TBARS were detected in EDTA propofol emulsions. Incubation (37°C, up to 6 h) of sulfite propofol emulsions in air resulted in further increases in TBARS (35–160%). No increases occurred in incubated EDTA propofol emulsions. Metabisulfite (0.25 mg/ml) alone added to 10% soybean oil resulted in large increases in TBARS that were inhibited in part by propofol (10 mg/ml) and completely by ascorbic acid (0.05 mg/ml). Soybean oil emulsion pH declined rapidly on the addition of metabisulfite (0.25 mg/ml). The addition of propofol (10 mg/ml) partially inhibited the decline in pH and ascorbic acid (0.05 mg/ml) completely inhibited it.

Conclusion: These results show that sulfite supports the peroxidation of lipids in soybean oil emulsions and propofol functions to partially inhibit these processes.

SULFITE (SO_3^{2-}) is added to a number of drug preparations as an antioxidant and antimicrobial agent. Recently, it has been included in a commercial propofol emulsion in the form of sodium metabisulfite ($\text{Na}_2\text{S}_2\text{O}_5$), which creates a unique sulfite-lipid drug formulation (propofol injectable emulsion 1% prescribing information, Gensia Sicor Pharmaceuticals Inc, Irvine, CA). Whether initially added to a solution as metabisulfite ($\text{S}_2\text{O}_5^{2-}$), bisulfite (HSO_3^-) or sulfite (SO_3^{2-}), the preservative activities of sulfites, antioxidant and antimicrobial effects, are a function of the sulfite ion.¹ Antioxidant effects of sulfite are due to its ability to serve as a reductant. It can undergo a one-electron oxidation, form-

ing the sulfite radical ($\text{SO}_3^{2-} \rightarrow \text{SO}_3^{\cdot-}$), or a two-electron oxidation, forming sulfate ($\text{SO}_3^{2-} \rightarrow \text{SO}_4^{2-}$).² The latter reaction causes solution acidification. Antimicrobial activities of sulfite are thought to be due to the release from sulfite of sulfur dioxide ($\text{SO}_3^{2-} \rightarrow \text{SO}_2$).³ Sulfur dioxide is a lipid-soluble gas that is capable of permeating cellular membranes, reforming sulfite, and undergoing intracellular oxidation.

In addition to its antioxidant properties, sulfite has been shown to exhibit prooxidant effects in certain conditions. Kaplan *et al.*⁴ reported that the addition of sulfite to an emulsion containing corn oil resulted in the oxidation of lipids as measured by the formation malondialdehyde and conjugated dienes, products of unsaturated lipid oxidation. Southerland *et al.*⁵ demonstrated that sulfite added to emulsions of the unsaturated fatty acids, linolenic acid (18:3) and arachidonic acid (20:4), resulted in lipid spectral changes indicative of lipid peroxidation and the formation of sulfite-lipid adducts. Lavoie *et al.*⁶ showed that sulfite in the presence of organic peroxides readily oxidized scopoletin, a coumarin derivative.

Current commercial propofol emulsions consist of 10% soybean oil emulsion (homogenized egg yolk phospholipid, 12 mg/ml; soybean oil, 100 mg/ml; and glycerol, 22.5 mg/ml), plus propofol (10 mg/ml), and either EDTA (0.05 mg/ml) or metabisulfite (0.25 mg/ml) as the antimicrobial agent.⁷ The evidence that sulfite can catalyze oxidation suggests that sulfite has the potential to induce lipid peroxidation in propofol emulsions. Susceptible unsaturated lipids are present in these formulations as fatty acid constituents of soybean oil triglycerides and phospholipid.^{8,9} On the other hand, propofol has antioxidant properties^{10,11} and is present in relatively high quantities in these emulsions. The objectives of the current study are to determine if lipid peroxidation occurs in sulfite propofol emulsions and to clarify the role of sulfite in this process.

Methods

EDTA propofol emulsions were obtained from AstraZeneca Pharmaceuticals (Wilmington, DE), and metabisulfite propofol emulsions were obtained from Gensia Sicor Pharmaceuticals (Irvine, CA). Ten percent soybean oil emulsion was obtained from Fresenius Kabi Clayton, LP (Clayton, NC). The emulsions were stored within the specified guidelines until use. All emulsions were used before their expiration date, except where noted. 2,6-Diisopropylphenol (propofol) and 5,5'-dithiobis(2-nitro-

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benzoic acid) were purchased from Aldrich Chemicals (Milwaukee, WI). Sodium metabisulfite, disodium edetate (EDTA), and ascorbic acid were from Fisher Scientific Inc. (Pittsburgh, PA).

Lipid peroxidation was assayed as thiobarbituric acid reactive substances (TBARS) by a modified thiobarbituric acid (TBA) method.¹² Samples of 1 ml of each emulsion were taken from intact commercially prepared vials, or from emulsions having been incubated, and immediately mixed with 10 mg ascorbic acid. The emulsions were cracked and lipids separated from the aqueous phase with three volumes of chloroform/methanol (2/1, vol/vol). After centrifugation, 1 ml of the aqueous phase was added to 1 ml 1% TBA solution and heated (60°C for 2.5 h). Samples were read at 532 nm. Verification that TBARS were not formed by sulfite during the assay process was made by routinely adding metabisulfite (0.25 mg/ml) to emulsions not containing metabisulfite prior to the TBARS assay. Nondetectable TBARS were defined by 532 nm absorbances in the samples that were no greater than that of deionized water. Standard curves were prepared by adding known quantities of malondialdehyde to EDTA propofol emulsion. EDTA propofol emulsions did not show detectable TBARS in this study and were suitable for constructing standard curves. Malondialdehyde was used to standardize TBARS. Malondialdehyde was obtained by adding malonaldehyde bis-(dimethyl acetal) to a 20% trichloroacetic acid solution and heating for 30 min at 60°C for acetal hydrolysis.

The aqueous phase of some samples containing the 532-nm absorbing TBA adduct was analyzed on a ThermoFinnigan Surveyor high-performance liquid chromatography system equipped with a Supelco Discovery HS column (75 × 2.1 mm ID, 3 μ m). The analyses were conducted by injecting 5 μ l of sample onto the high-performance liquid chromatography column using 5 mM ammonium acetate/methanol (70/30, vol/vol) as mobile phase at a flow rate of 400 μ l/min. Each eluting fraction was scanned from 300 to 600 nm using a photodiode array detector.

Emulsion sulfite was measured by a modified 5,5'-dithiobis-(2-nitrobenzoic acid) method.¹³ One milliliter of emulsion was treated with 100 μ l of 10% NaCl solution, and the lipids and propofol were extracted with 2 ml of chloroform/methanol (2/1, vol/vol). The upper aqueous phase was removed and added to 4.6 ml of a 0.001 M EDTA solution. One milliliter of the EDTA-containing mixture was diluted with 1 ml pH 7 buffer (VWR pH 7 buffer solution). Then 0.5 ml of a buffered solution of 5,5'-dithiobis-(2-nitrobenzoic acid) was added. The 5,5'-dithiobis-(2-nitrobenzoic acid) solution consisted of 0.2 g of 5,5'-dithiobis-(2-nitrobenzoic acid) and 1 ml of Fisher reagent grade ethanol diluted to 50 ml with VWR pH 7 buffer solution. The mixture was allowed to stand in the dark at room temperature for 30 min. The reaction mixture was diluted with 7.5 ml deionized water, and

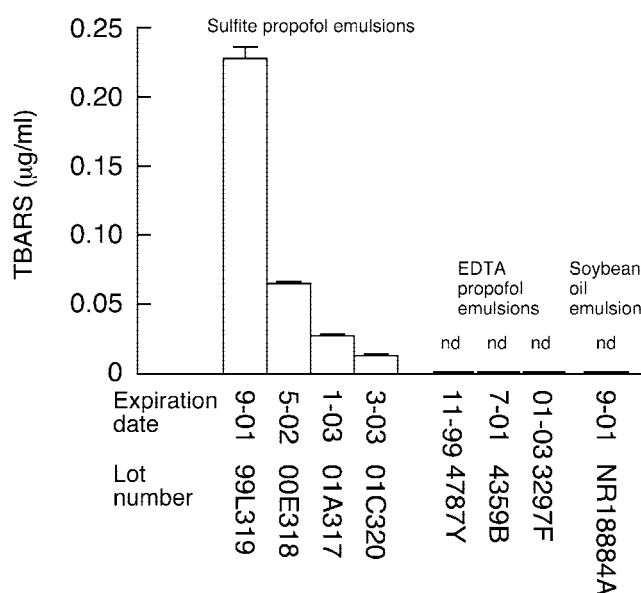


Fig. 1. Thiobarbituric acid reacting substances (TBARS) in commercial metabisulfite and EDTA-containing propofol emulsions. Assays were performed in triplicate (mean \pm SD) from single bottles taken at random from a case of each lot. All assays were performed simultaneously and prior to the expiration of all emulsions except the EDTA propofol emulsion lot 4787Y (11–99).

the absorbances were read at 412 nm. Standard curves were constructed by adding known quantities of sodium metabisulfite to EDTA propofol emulsions. Standard sodium metabisulfite solutions were made fresh and immediately used. Emulsion pH measurements were made with standardized pH electrodes at room temperature.

Emulsion incubations were performed by placing samples in a Dubnoff metabolic incubator at 37°C and shaking (120 cycles/min) with emulsion exposure to air. A simulated intravenous drip was performed by hanging spiked 50-ml vials of sulfite propofol at room temperature and releasing 3.5 ml of emulsion at each hour for TBARS analysis.

Statistical analyses were performed by paired *t* test or repeated-measures analysis of variance (Scheffé *F* test). *P* \leq 0.05 was considered significant.

Results

Thiobarbituric acid reacting substance concentrations in properly stored intact vials of sulfite propofol emulsion and EDTA propofol emulsion are shown in figure 1. Each vial of sulfite propofol emulsion showed the presence of TBARS, whereas the EDTA propofol emulsion contained no detectable TBARS. Ten percent soybean oil emulsion also did not show detectable concentrations of TBARS. The quantities of TBARS in sulfite propofol emulsion showed high degrees of variability where concentrations appeared to positively correlate with emulsion age as based on emulsion expiration date.

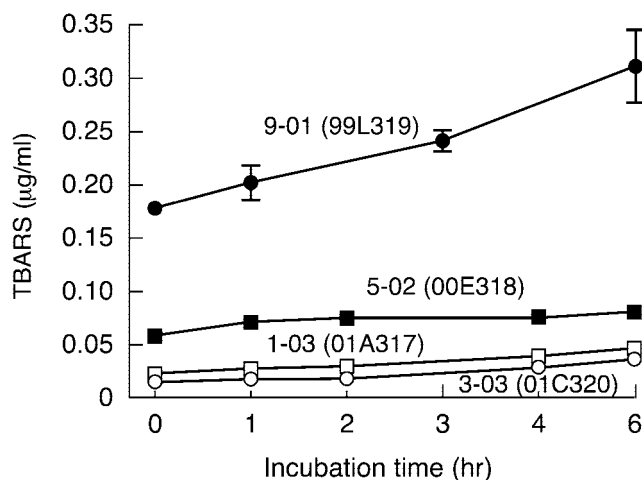


Fig. 2. Thiobarbituric acid reacting substances (TBARS) in commercial propofol emulsions on aerobic incubation. One-milliliter samples of emulsion were incubated at 37°C with shaking. Values are means (\pm SD) of triplicate determinations. Labels are sulfite propofol emulsion expiration date and lot number. EDTA-containing propofol emulsions (expiration date, lot number: 01-03, 3297F; 7-01, 4359B; 11-99, 4787Y) did not yield detectable TBARS on incubation.

Thiobarbituric acid reacting substances in sulfite and EDTA propofol emulsions incubated in air at 37°C are shown in figure 2. Emulsions were incubated to facilitate sulfite reactivity. TBARS levels at 0 h confirm the initial relative concentrations of TBARS in the different manufactured lots of metabisulfite propofol emulsion. Concentrations slowly increased (35–160%) in the sulfite propofol emulsions during the 6-h incubation period. TBARS in the incubated emulsions also showed a high degree of variability. The increased levels following incubation likewise correlated with emulsion expiration date. The EDTA propofol emulsions did not yield detectable TBARS at any incubation time.

The formation of TBARS in sulfite propofol emulsions at room temperature during a simulated 12-h intravenous drip at room temperature is shown in figure 3. TBARS were slower in forming than on incubation but increased at 8–12 h.

The role of sulfite in TBARS formation in propofol emulsions was examined by adding various emulsion components individually or in combination to 10% soybean oil emulsion. Figure 4 shows that metabisulfite alone added to emulsion caused a large increase in TBARS during a 90-min incubation period. The addition of propofol (10 mg/ml) inhibited the formation of TBARS. EDTA, either alone, in the presence of metabisulfite, or in the presence of metabisulfite and propofol, did not substantially influence TBARS formation.

The stability of sulfite in soybean oil emulsions is shown in figure 5. Sulfite (0.25 mg metabisulfite per milliliter of initial concentration) in 10% soybean oil emulsion declined rapidly on incubation and was nearly depleted in 4 h. The addition of propofol (10 mg/ml) to

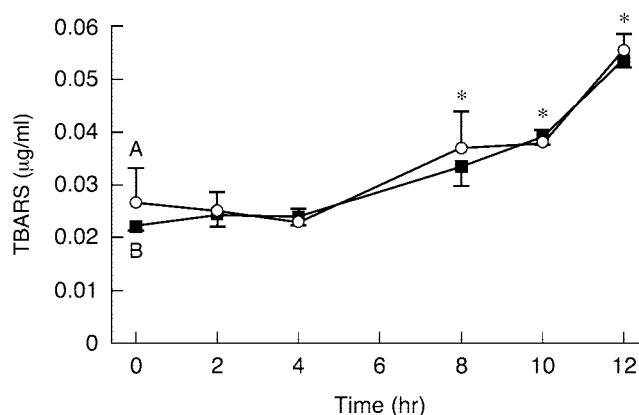


Fig. 3. Thiobarbituric acid reacting substances (TBARS) in sulfite propofol emulsion during a simulated 12-h intravenous drip at room temperature. Two vials (A and B) of sulfite propofol emulsion (both 08/03, 010K310) were dripped over a period of 12 h and emulsion collected and analyzed as described in Methods. Values are the means (\pm SD) of triplicate determinations. *Values significantly different than 0-h values.

the emulsion containing sulfite slowed the loss of sulfite. However, sulfite concentrations still dropped approximately 30% in 6 h in the presence of propofol. The addition of a low amount of ascorbic acid (0.05 mg/ml) to the propofol-containing emulsion completely inhibited sulfite loss.

While the addition of propofol to metabisulfite containing 10% soybean oil emulsion did not result in an increase in TBARS during a 90-min incubation period, increased TBARS was detected in these propofol emul-

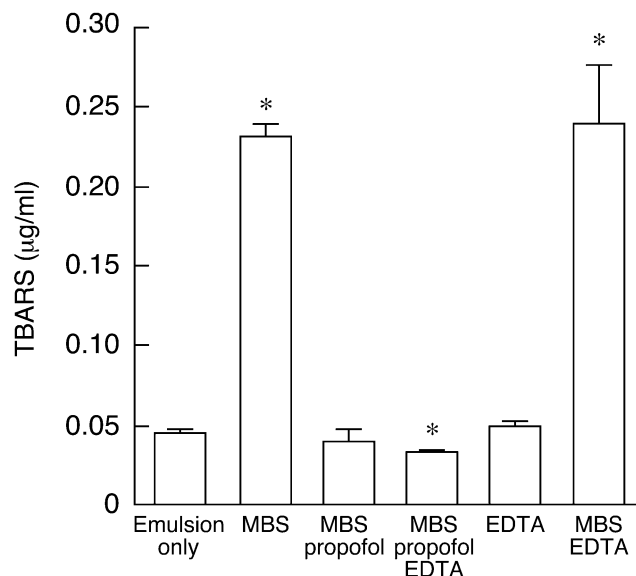


Fig. 4. The effect of propofol emulsion components on thiobarbituric acid reacting substances (TBARS) in 10% soybean oil emulsion. Samples were incubated for 90 min at 37°C. Emulsions contained, where indicated, 0.25 mg/ml metabisulfite (MBS), 10.0 mg/ml propofol, and 0.05 mg/ml EDTA. Values are the means (\pm SD) of triplicate determinations. *Values significantly different from soybean oil emulsion with no added components.

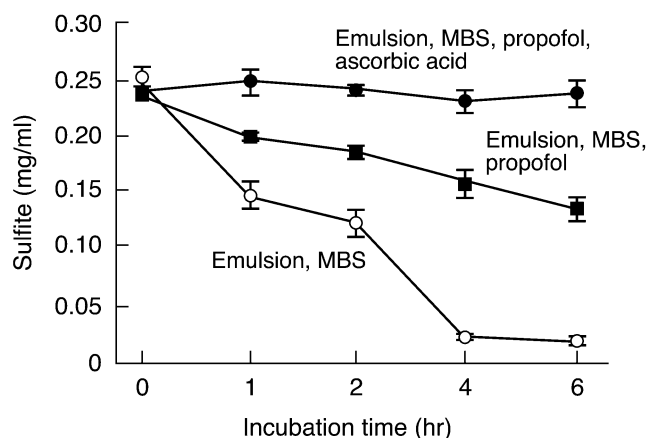


Fig. 5. The effect of propofol and ascorbic acid on sulfite stability in 10% soybean oil emulsions. Samples were incubated at 37°C for the times noted and contained, where indicated, 0.25 mg/ml metabisulfite (MBS), 10 mg/ml propofol, and 0.05 mg/ml ascorbic acid. Values are the means (\pm SD) of triplicate determinations.

sions on incubation for 4 and 6 h (fig. 6). This indicates that sulfite in the reconstituted propofol emulsion will generate TBARS in time. The absence of propofol allowed a large rapid increase in TBARS that continued to increase during the 6-h incubation.

The effect of sulfite on emulsion pH is shown in figure 7. The addition of sulfite caused a small initial decrease in pH consistent with the weakly acidic properties of sulfite. On incubation, pH progressively declined during 6 h, causing substantial emulsion acidification (to pH 3.3). The addition of 10 mg/ml propofol to these emulsions, which yields an emulsion having the same contents as the commercial sulfite propofol emulsion, also showed a reduction in pH over time; however, propofol partially inhibited acidification (to pH 4.6 in 6 h). The addition of propofol and ascorbic acid completely inhibited the sulfite-dependent decrease in pH.

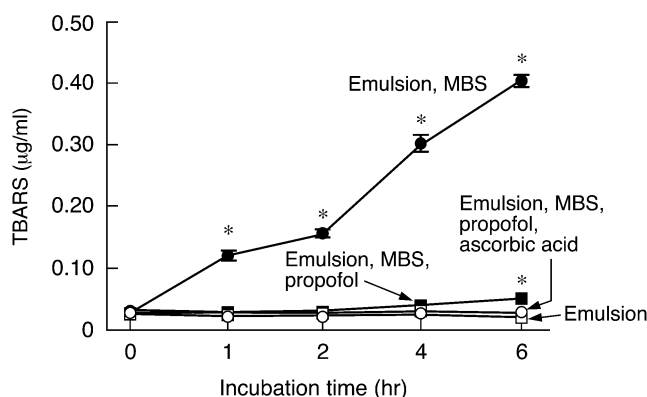


Fig. 6. Thiobarbituric acid reacting substances (TBARS) in soybean oil emulsions containing metabisulfite, propofol, or ascorbic acid. Emulsions were incubated at 37°C for the time periods noted and contained, where indicated, 0.25 mg/ml metabisulfite (MBS), 10 mg/ml propofol, and 0.05 mg/ml ascorbic acid. Values are the means (\pm SD) of triplicate determinations. *Values significantly greater than 0-h values in the same emulsion.

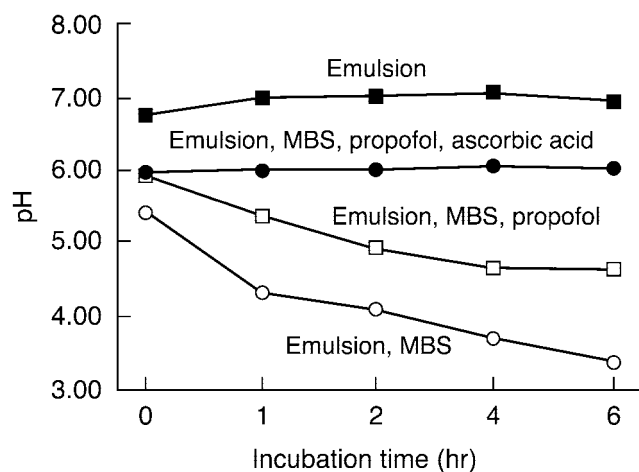


Fig. 7. The effect of metabisulfite (MBS), propofol, or ascorbic acid on soybean oil emulsion pH. Emulsions were incubated at 37°C for the time periods noted and contained, where indicated, 0.25 mg/ml metabisulfite, 10 mg/ml propofol, and 0.05 mg/ml ascorbic acid. Values represent single sequential determinations.

Analysis of the 532-nm absorbing TBA adduct by high-performance liquid chromatography showed that the adducts formed in the sulfite propofol emulsion and 10% soybean oil emulsions chromatographed identically with the TBA adduct generated with authentic malondialdehyde. Furthermore, the 532-nm absorbing TBA adducts formed from sulfite propofol emulsion and 10% soybean oil emulsion containing sulfite had identical uv-vis spectra as the malondialdehyde adduct, indicating that the TBARS measured in this study is predominantly malondialdehyde.

Discussion

This study demonstrates that the oxidation of sulfite in 10% soybean oil emulsion facilitates the peroxidation of emulsion lipids. This is shown by the fact that sulfite alone added to emulsions caused a large increase in TBARS formation, and that both propofol and ascorbic acid inhibited TBARS, depletion of sulfite, and the decrease in emulsion pH, the latter of which is caused by the conversion of sulfite to sulfate.²

The utility of measuring TBARS, in particular malondialdehyde, is that malondialdehyde is an end-product of lipid peroxidation.¹⁴ Lipid hydroperoxides, cycloperoxides, and conjugated dienes are subject to further reaction with sulfite.^{6,15} Malondialdehyde results from the peroxidation of fatty acids containing three or more unsaturations.¹⁴ In soybean oil emulsions, it largely results from the peroxidation of linolenic acid (18:3) and thus is a minor peroxidation product. Ten percent soybean oil emulsion and the soybean oil-based propofol emulsions have similar fatty acid contents consisting of (by weight) linolenic acid (18:3, 8%), palmitic acid (16:0,

13%), stearic acid (18:0, 4%), oleic acid (18:1, 22%), and linoleic acid (18:2, 52%).¹⁶

Mechanisms of sulfite lipid peroxidation have been proposed by other investigators and are thought to initially involve sulfite oxidation to the reactive sulfite radical ($\text{SO}_3^{-2} \rightarrow \text{SO}_3^{\cdot-}$).¹⁷⁻¹⁹ The sulfite radical may subsequently react with oxygen to form oxidized sulfite species, such as the sulfite peroxy and sulfate anion radicals,^{2,20} which in turn react with lipid. Alternatively, sulfite radicals may directly react with lipids, abstracting hydrogens, and forming lipid radicals that react with molecular oxygen. Propagating reactions include the reaction of sulfite with lipid peroxides ($\text{LOOH} + \text{SO}_3^{-2} \rightarrow \text{LO}^{\cdot} + \text{SO}_3^{\cdot-}$; $\text{L} = \text{lipid}$), and the reaction of sulfite with lipid radicals ($\text{L}^{\cdot} + \text{SO}_3^{-2} \rightarrow \text{SO}_3^{\cdot-} + \text{LH}$), both reactions of which generate more sulfite radical.¹⁷

Low amounts of antioxidant tocopherols (α and γ) are naturally present in soybean oil emulsions as components of soybean oil.²¹ However, these tocopherol compounds, either due to their low quantities or to the oxidative potency of sulfite, nevertheless allow significant sulfite catalyzed lipid peroxidation to proceed. The effect of propofol in inhibiting malondialdehyde formation is consistent with the antioxidant properties of propofol. As an alkyl substituted phenol, propofol has an ability to scavenge free radicals.^{10,11} It may inhibit TBARS formation at any point in the reaction sequence following sulfite radical formation,²² but the finding that propofol inhibited TBARS, sulfite depletion, and acidification suggests that it may have a direct inhibitory effect on sulfite oxidation.

It was an unexpected finding that the addition of a small quantity of ascorbic acid (0.05 mg/ml) would completely inhibit TBARS, sulfite depletion, and acidification in the propofol sulfite emulsions. Ascorbic acid (vitamin C) can function as a water-soluble antioxidant.^{23,24} Its high degree of effectiveness in inhibiting these processes may relate to it predominantly localizing in the aqueous phase of the emulsion, as does sulfite, due to its high water solubility. Conversely, the lesser ability of propofol to inhibit sulfite catalyzed lipid peroxidation may be a consequence of the high lipophilicity of propofol, which causes it to mostly reside in the emulsified soybean oil microdroplets as opposed to the aqueous phase,⁷ or it may relate to a lower antioxidant potential of propofol. Because of differences in reactant solubilities (lipid *vs.* sulfite), sulfite catalyzed lipid peroxidation in these emulsions may occur primarily at the lipid-aqueous phase interface.

While organic peroxides readily react with sulfite, causing oxidant activity, the precise role of lipid peroxides in sulfite lipid reactivity in these emulsions is not clear. As noted above, sulfite is known to act as a lipid antiperoxide by cleaving hydroperoxides to the corresponding oxyl radicals ($\text{LOOH} + \text{SO}_3^{-2} \rightarrow \text{LO}^{\cdot} + \text{SO}_3^{\cdot-}$), thus initiating free radical formation.⁶ However, lipid

peroxides (cyclo and hydroperoxides) are prerequisite to malondialdehyde formation.¹⁴ Therefore, sulfite appears to have a biphasic effect of generating and depleting lipid peroxides, the balance of which may be determined by relative sulfite concentration.

The detection of TBARS in the commercially prepared sulfite propofol emulsions confirms that propofol does not abolish the sulfite lipid peroxidation. Furthermore, it indicates that either during the manufacturing process or during storage, variable low degrees of lipid peroxidation occur. The apparent correlation of TBARS with emulsion age suggests that lipid peroxidation occurs during emulsion storage. The oxygen source may be residual oxygen not removed by emulsion purging with nitrogen during the manufacturing process.

The consequences of propofol emulsion lipid peroxidation remain to be determined. Lipid peroxidation in soybean oil emulsions used in parental nutrition is thought to be detrimental due to the ability of lipid peroxides to cause free radical-dependent damage.^{24,25} More recent studies of malondialdehyde and 4-hydroxynonenal, the latter of which is a product of the peroxidation of linoleic acid, have shown that these aldehydic compounds are reactive with biologic macromolecules.²⁶⁻²⁸ While the experiments to elucidate the role of sulfite in emulsion lipid peroxidation in this study were performed at physiologic temperature, the relevance of the lesser lipid peroxidation in sulfite propofol emulsions exposed to air at room temperature is unknown.

Sulfite reactivity in emulsions is not confined to lipid peroxidation.²⁹ Sulfite has been shown to cause the oxidation of propofol in emulsions resulting in a propofol dimer,³⁰ thus directly effecting the drug substance. Sulfite is also reactive *via* other mechanisms toward lipids. It can form lipid adducts by reacting with lipid unsaturations and with lipid peroxides to form sulfonated lipids.¹⁵ For these reasons, the reactions of sulfite in propofol emulsions during clinical use, as well as sulfite reactivity *in vivo* on propofol emulsion administration, needs to be further studied.

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