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THE ANTIOXIDANT POTENTIAL OF PROPOFOL (2,6-DIISOPROPYLPHENOL)†

P. G. MURPHY, D. S. MYERS, M. J. DAVIES, N. R. WEBSTER AND J. G. JONES

SUMMARY

We have examined in vitro *the antioxidant properties of 2,6-diisopropylphenol. In studies using electron spin resonance spectroscopy we have demonstrated that 2,6-diisopropylphenol acts as an antioxidant by reacting with free radicals to form a phenoxyl radical—a property common to all phenol-based free radical scavengers. In additional experiments, the antioxidant properties of the clinical formulation of 2,6-diisopropylphenol (propofol) have been measured using an assay of antioxidant potential. In these experiments, propofol, but not Intralipid, was found to exhibit significant antioxidant activity, such that in the range of propofol concentrations examined (10^{-6}–10^{-5} mol litre^{-1}), each molecule of 2,6-diisopropylphenol was able to scavenge two radical species. We conclude that the free radical scavenging properties of 2,6-diisopropylphenol resemble those of the endogenous antioxidant α-tocopherol (vitamin E).*

KEY WORDS

Anaesthetics, intravenous. propofol, antioxidant potential.

Free radicals are believed to contribute to the tissue injury associated with a wide range of acute and chronic conditions [1]. Of particular interest to the anaesthetist and intensive therapist are reperfusion injury and acute inflammatory processes such as acute lung injury, severe sepsis and multisystem organ failure [2]. Free radical scavengers, or antioxidants, represent an important component of the body's defences against such free radical mediated injury, so that boosting of these defences may emerge as an important therapeutic aim. Although specific antioxidant therapies are still at the experimental stage, several drugs are already available which, incidental to their major biological action, may augment free radical scavenging capacity. This group of drugs includes salicylates [3] and barbiturates [4], although the clinical value of such properties is uncertain.

Propofol, the currently available formulation of the anaesthetic agent 2,6-diisopropylphenol, has been shown to attenuate experimental reperfusion injury in the cerebral cortex [5], although the mechanism underlying this action has yet to be defined. Oxygen-derived free radicals may contribute to such injury, and as propofol is chemically similar to phenol-based free radical scavengers such as butylated hydroxytoluene (BHT) and the endogenous antioxidant vitamin E (fig. 1) [1], we have examined the hypothesis that propofol is a free radical scavenger.

Phenol-based antioxidants (R-OH) scavenge free radical species (X·) by a process of hydrogen abstraction, and thereby themselves become a (less reactive) phenoxyl radical (R-O·):

$$\text{R-OH} + \text{X}\cdot \rightarrow \text{R-O}\cdot + \text{XH}$$

In order to examine the hypothesis that propofol behaves in a similar way, we have used electron spin resonance (ESR) spectroscopy, one of the more specific methods of characterizing radical reactions [1], to examine the way in which propofol reacts with free radical species, and in particular to identify if a propofol-derived phenoxyl radical is generated under such conditions.

Lipid peroxidation is a free radical-mediated chain reaction [1]. In the second part of this *in vitro* study we have measured the free radical scavenging capacity of propofol by comparing its ability to arrest lipid peroxidation with that of an established free radical scavenger of known potency.

METHODS

In vitro *investigation of the oxidation of propofol*

As noted above, the chemical similarities between 2,6-diisopropylphenol and phenol-based antioxidants suggest that, after oxidation by a free radical species, it should become a phenoxyl radical. This hypothesis was tested in two parts.

The first experiments were designed to define the ESR spectrum of the oxidation product of 2,6-diisopropylphenol (the propofol phenoxyl radical). The limited aqueous solubility of this material required that these experiments were performed both using a saturated solution of 2,6-diisopropylphenol (Sigma) dissolved in a 2:1 mixture of water and acetone, and on the clinical formulation of the

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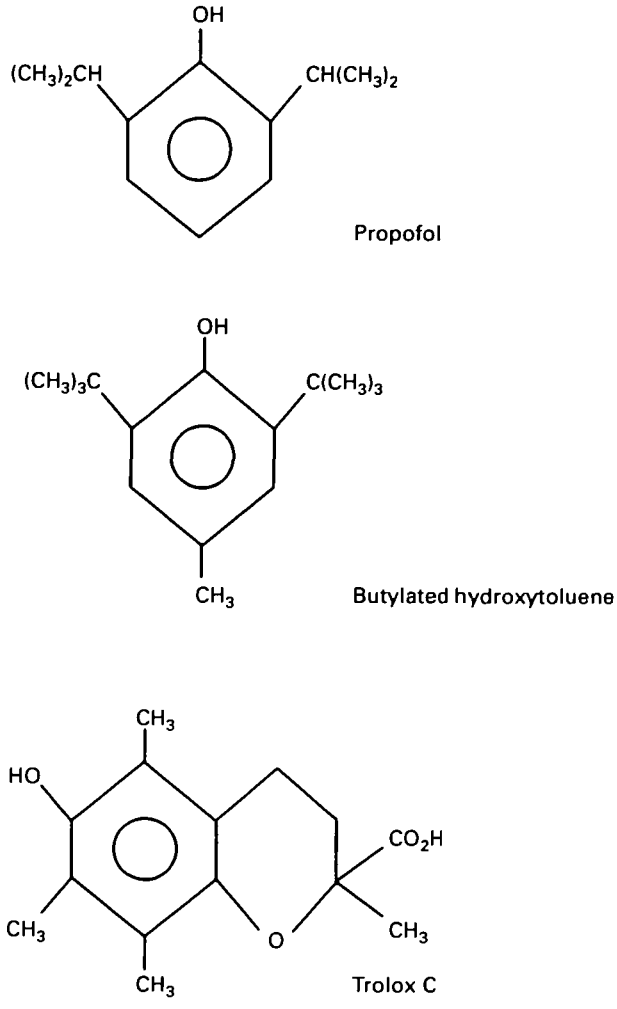


FIG. 1. Structural formulae of 2,6-diisopropylphenol (propofol), butylated hydroxytoluene (BHT) and Trolox C (a water soluble analogue of vitamin E). All three compounds carry a hydroxyl substituent on their phenol rings, which is known to confer free radical scavenging properties on both BHT and Trolox C.

compound—propofol. Oxidation was carried out with potassium permanganate 1 mmol litre^{-1} (pH range 9.5–11.5), using a two-stream rapid mixing system as described previously [6, 7]. ESR spectra of the reaction mixtures were recorded at room temperature using a Bruker ESP 300 ESR spectrometer equipped with 100-kHz modulation and a Bruker ER035M gaussmeter for field calibration. We measured directly from the field scan the hyperfine coupling constants characterizing the radicals which were detected.

When the nature of the propofol phenoxyl radical had been identified, additional experiments were designed to investigate if the same species was produced when propofol was exposed to radical generating systems—that is, to identify if propofol was able to scavenge these radicals. Two radical generating systems were used: a mixture of haematin 250 μmol litre^{-1} and butyl hydroperoxide (^{t}BuOOH) 3.5 mmol litre^{-1}, which generates peroxyl and alkoxyl radicals, and a hydroxyl radical (OH·) generating system consisting of titanium triiodide 8 mmol litre^{-1} and hydrogen peroxide 33 mmol litre^{-1}. These were applied to three preparations of 2,6-diisopropylphenol (all at pH 7): a saturated aqueous solution of 2,6-diisopropylphenol; a saturated solution of 2,6-diisopropylphenol in a 2:1 mixture of water and acetone, and the clinical formulation of propofol; the reactions were examined under the same ESR conditions as defined above, but with the use of a three-stream rather than two-stream rapid mixing system [8].

In vitro *measurement of antioxidant potential*

We have investigated the ability of propofol to arrest lipid peroxidation using the Total Radical Antioxidant Potential (TRAP) assay [9]. This is an assay of the antioxidant activity of plasma, which is obtained by comparing the duration of time for which plasma is able to resist lipid peroxidation with that achieved after addition of a known amount of an established antioxidant—in this case Trolox C (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (fig. 1)), a water soluble analogue of vitamin E. Lipid peroxidation was initiated by adding a small quantity of a water soluble azo compound 2,2-azo-bis-(2-amidinopropane) (ABAP) to a sample of diluted plasma. As lipid peroxidation is an oxygen consuming reaction, the rate of peroxidation was estimated by following changes in the oxygen tension (Po_2) in the reaction chamber with time. Initially, antioxidants present within the plasma limit the rate of peroxidation, but as they are consumed by the scavenging process, lipid peroxidation is able to take place more readily. A plot of the change in Po_2 of the system with time (fig. 2) thus shows a gradual increase in the rate of decline of oxygen tension as the free radical-mediated oxygen-consuming reactions are able to take place at an increasing rate. The peroxidation reactions can be inhibited by adding exogenous antioxidant to the system (in these experiments, Trolox C), but this effect too is only temporary, after which the rate of oxygen consumption once again increases. As displayed in figure 2 by fitting tangents to those parts of the Po_2–time curves where the rate of change in Po_2 is at its least (immediately after the addition of ABAP or Trolox C) and at its greatest (when all available antioxidants have been consumed), these plots are used to derive graphically the "induction" times for plasma and Trolox C, τ_P and τ_{Tr} respectively. These induction times were then used to quantify the antioxidant activity of the plasma in terms of a molar TRAP value, which is calculated from the equation:

$$\text{TRAP} = \frac{n[\text{Trolox}].\tau_P}{f.\tau_{Tr}}$$

where n is the number of radicals trapped per molecule of Trolox (= 2), and f is a factor which allows for the dilution of the plasma. Although the fitting of the tangents introduces a subjective element to the method, the results from this technique correspond well with a more objective but technically more demanding assay of plasma antioxidant potential [9].

Measurement of plasma TRAP. Changes in oxygen tension in the reaction system were monitored using a Rank oxygen electrode (Rank Brothers, Bottisham, Cambridge), with the Clark-type electrode mounted in the base of a reaction cell and maintained at 37 °C

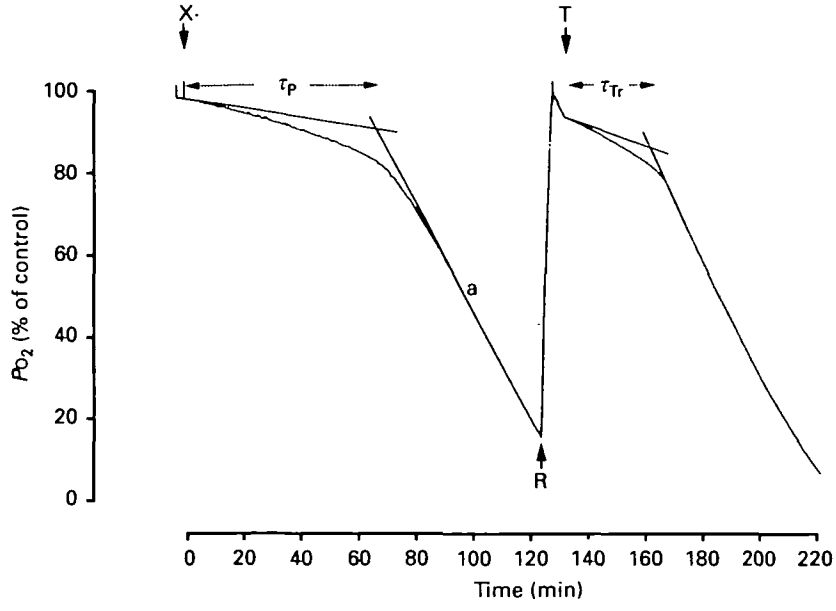


FIG. 2. Changes in oxygen tension (Po_2) with time observed using the TRAP assay of plasma antioxidant capacity. ABAP (X·) was added to initiate lipid peroxidation. At a, the rate of decrease in Po_2 reached a maximum as a result of consumption of available antioxidants in the system and consequent increase in the rate of (oxygen consuming) lipid peroxidation. Following partial reoxygenation (R), the addition of 25 µl of Trolox C 0.4 mmol litre^{-1} (T) temporarily arrested these reactions, so that the rate of oxygen consumption slowed again. The induction periods for plasma and Trolox C (τ_P and τ_{Tr}, respectively) were derived graphically by fitting tangents to the shallowest and steepest extreme of the appropriate Po_2–time curve. The total radical antioxidant potential (TRAP) value for the plasma was calculated from the ratio of the two induction periods.

with a heated water jacket. Thirty microlitre of ABAP 0.4 mol litre^{-1} (Polysciences) was added to 3 ml of aerated, prewarmed phosphate buffered saline 10 mmol litre^{-1} (Sigma). Fresh plasma (100-µl aliquots) donated on the day of the experiments by healthy, non-fasted human volunteers, was vortex mixed with 99% linoleic acid 9 µl (antioxidant free, Sigma) for 30 s and 50 µl of the resulting mixture added to the cell. Linoleic acid was stored in the dark at −40 °C under nitrogen, and used within 1 week. After completion of the plasma induction period, the cell was re-oxygenated before adding 25 µl of Trolox C 0.4 mmol litre^{-1} (Aldrich).

Measurement of the antioxidant capacity of propofol. The ability of propofol to arrest lipid peroxidation in this system was assessed in two ways. In the first series of experiments, after measurement of plasma TRAP as described above, propofol (the clinical formulation of 2,6-diisopropylphenol in 10% Intralipid: Diprivan, ICI) was added to the *in vitro* system in the same way as Trolox C. The effect of propofol was examined at three different concentrations (1×10^{-6} mol litre^{-1}, 5×10^{-6} mol litre^{-1} and 1×10^{-5} mol litre^{-1}; $n = 8$ in each case), whilst in parallel control experiments the effect of equivalent amounts of Intralipid (10% Intralipid solution, KabiVitrum) was determined. Induction times for Trolox C and propofol were then derived from the plots of Po_2 against time at these three propofol concentrations. As it is known that each molecule of Trolox C traps two radicals produced in this system ($n_{Tr} = 2$), these data were used to calculate a similar value for propofol, according to the equation:

$$n_{prop} = n_{Tr} \cdot \left(\frac{C_{Tr}}{C_{prop}}\right) \cdot \left(\frac{\tau_{prop}}{\tau_{Tr}}\right)$$

where C_{prop} and C_{Tr} are the concentrations of propofol and Trolox C, respectively, and τ_{prop} is the induction time for propofol.

In a second series of experiments, the contribution made by propofol to plasma antioxidant capacity was examined. Propofol was added to plasma obtained from healthy human volunteers to produce a concentration of 20 µg ml^{-1}, parallel control samples of plasma receiving equivalent volumes of Intralipid. The TRAP values for the two groups of samples were measured and the difference between the two groups taken to represent the contribution made by 2,6-diisopropylphenol to the antioxidant capacity of the plasma.

Statistical analysis

Where appropriate, values are expressed as mean (SEM). Statistical significance was assessed using Student's unpaired t test and accepted when $P < 0.05$. Significant differences were quantified by confidence interval analysis and expressed in terms of 95% confidence intervals of the difference of the mean.

RESULTS

ESR studies

Essentially identical ESR spectra were generated from the oxidation of 2,6-diisopropylphenol and propofol by potassium permanganate (fig. 3), although baseline noise was greater in the propofol experiments, presumably as a result of the presence of lipid. The parameters of this radical species (a_H 0.966, a_{2H} 0.344 and a_{2H} 0.172 mT) are consistent with the appearance of a phenoxyl radical following oxidation [10].

Weak spectra of the phenoxyl radical species identified above were detected after exposure of a

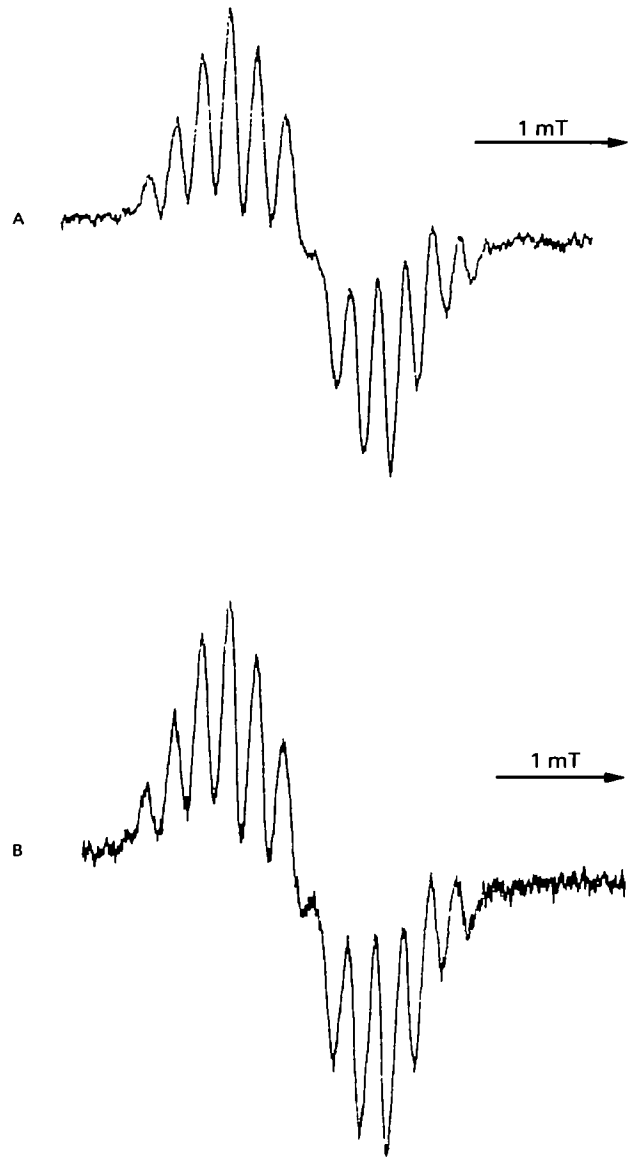


FIG. 3. Electron spin resonance (ESR) spectra of 2,6-diisopropylphenol adducts produced by the oxidation of a saturated solution of 2,6-diisopropylphenol in a 2:1 mixture of water and acetone (A) and propofol (B) with potassium permanganate 1 mmol litre^{-1}. The ESR characteristics of this radical identify it as a phenoxyl species. Gain 1×10^6 in each case; baseline noise greater in propofol spectrum as a result of the presence of lipid.

saturated solution of 2,6-diisopropylphenol in water–acetone to the haematin–^{t}BuOOH system, suggesting that 2,6-diisopropylphenol can scavenge peroxyl and alkoxyl radicals. The presence of lipid, however, interfered with parallel experiments using propofol. In the experiments using the titanium–hydrogen peroxide system, OH· appeared to react preferentially with the acetone or lipid solvents, while in experiments using the aqueous preparation the solubility of 2,6-diisopropylphenol was so poor that the small concentrations of radical produced were insufficient to allow ESR detection.

Antioxidant capacity of propofol

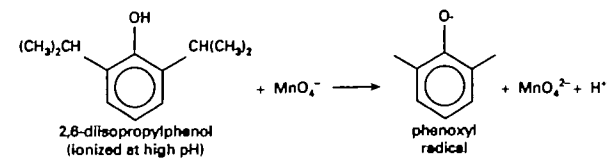
Comparison of the antioxidant potencies of propofol, Intralipid and Trolox C. Equivalent amounts of both Trolox C and propofol were able to decrease the rate of oxygen consumption (and therefore the rate of lipid peroxidation) to approximately the same extent in the TRAP assay system whilst, in contrast, the addition of an equivalent amount of Intralipid had no effect (fig. 4).

The induction times for the three concentrations of propofol examined are shown in table I, together with the corresponding induction times for Trolox C. As expected, the induction period for propofol, τ_{prop}, increased in proportion to the concentration of propofol used. The antioxidant potency of propofol, n_{prop}, was similar across the range of concentrations studied: 1.9 (SEM 0.1), 2.2 (0.1) and 2.1 (0.1) at 1×10^{-6}, 5×10^{-6} and 1×10^{-5} mol litre^{-1}, respectively.

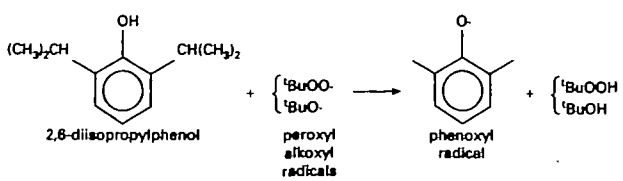
Effect of propofol on plasma TRAP. The addition of propofol to human plasma significantly increased the plasma antioxidant capacity. Thus the TRAP value measured for propofol-treated plasma was significantly greater than that after addition of Intralipid (1238 (30) μmol litre^{-1} compared with 1023 (27) μmol litre^{-1}, respectively; 95% confidence interval of the difference = 130–301 ($P < 0.0001$)).

DISCUSSION

Vitamin E is an important endogenous cell membrane antioxidant, while BHT is an established free radical scavenger used widely as a food additive to prevent rancidification of fats [1]. The chemical similarities between these phenol-based antioxidants and 2,6-diisopropylphenol suggest that the latter may behave in a similar fashion. ESR spectroscopy is a powerful tool for characterizing the nature of radical reactions, and has been used in this study first to confirm that 2,6-diisopropylphenol indeed becomes a phenoxyl radical when oxidized by permanganate:



Definition of the ESR spectrum of this phenoxyl species then allowed us to test the hypothesis that 2,6-diisopropylphenol was able to act as a free radical scavenger by looking for its production when the agent was exposed to radical generating systems. Identification of this species when 2,6-diisopropylphenol was exposed to a peroxyl–alkoxyl radical generating system (haematin–^{t}BuOOH) confirmed this hypothesis:



Failure to demonstrate a similar effect when 2,6-diisopropylphenol was exposed to hydroxyl radicals

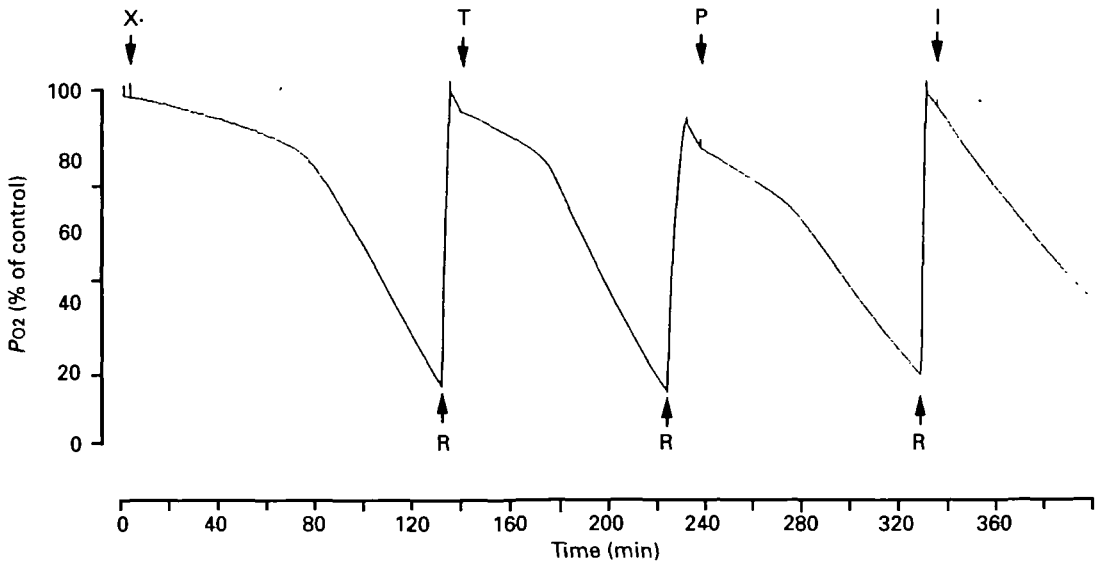


FIG. 4. Effects of Trolox C (T), propofol (P) and Intralipid (I) on the rate of oxygen consumption in the TRAP assay system. Trolox C and propofol (3.3×10^{-6} mol litre^{-1} in each case) arrested oxygen consumption (and so lipid peroxidation) to the same extent. In contrast, an equivalent amount of 10% Intralipid was without effect. X· = ABAP; R = reoxygenation.

TABLE I. *The antioxidant potency of propofol in the TRAP assay system. The mean (SEM) number of radicals scavenged per molecule of propofol (n_{prop}) is calculated from the ratio of the graphically derived induction periods for Trolox C and propofol (τ_{Tr} and τ_{prop}, respectively). The effect of propofol was examined at three different concentrations (1×10^{-6}, 5×10^{-6} and 1×10^{-5} mol litre^{-1}) and compared with a standard Trolox C concentration (3.3×10^{-6} mol litre^{-1})*

	Propofol concentration (mol litre^{-1})		
	1×10^{-6}	5×10^{-6}	1×10^{-5}
τ_{Tr} (min)	31.3 (0.7)	31.3 (0.6)	27 5 (1.7)
τ_{prop} (min)	8.9 (0.3)	52.1 (1.3)	85.0 (3.1)
n_{prop} (No.)	1.9 (0.1)	2.2 (0.1)	2 1 (0.1)

was disappointing, but not surprising, with the hydroxyl radical reacting preferentially with the large concentrations of acetone present as a solvent. In much the same way, as free radicals are known to react with lipid, it is no surprise that parallel experiments using the clinical formulation propofol were inconclusive. Nevertheless, these ESR spectroscopic studies have served to establish qualitatively the nature of the possible interaction between 2,6-diisopropylphenol and free radicals.

In the second part of the study, we have attempted to quantify the antioxidant potency of 2,6-diisopropylphenol by examining its ability to limit lipid peroxidation. The demonstration that propofol, but not Intralipid, arrested oxygen consumption in the TRAP assay supports the hypothesis that 2,6-diisopropylphenol is a free radical scavenger, and we would propose that it interferes with lipid peroxidation in the following way. Lipid peroxidation is initiated according to the reaction:

$$LH + X\cdot \rightarrow L\cdot + XH$$

in which a radical X· (such as the carbon-centered radical generated by ABAP) abstracts a hydrogen atom from lipid (LH) to produce an intermediate lipid radical (L·). This radical is able to combine with molecular oxygen to form a lipid peroxyl radical (L-OO·), which is capable of abstracting a hydrogen atom from another lipid molecule:

$$L\cdot + O_2 \rightarrow L\text{-}OO\cdot$$
$$L\text{-}OO\cdot + LH \rightarrow L\cdot + L\text{-}OOH$$

After initiation, lipid peroxidation is a self-propagating chain reaction producing lipid hydroperoxides (L-OOH). On the basis of this study, we suggest that 2,6-diisopropylphenol (PH) interferes with lipid peroxidation via a process of hydrogen abstraction:

$$L\text{-}OO\cdot + PH \rightarrow P\cdot + L\text{-}OOH$$

The phenoxyl radical species which is thereby generated (P·, the phenoxyl radical which we have identified in these ESR experiments) is not reactive enough to propagate lipid peroxidation itself as a result of the free electron of this species being distributed throughout the phenol ring of the compound, and lipid peroxidation is therefore arrested. The TRAP assay experiments demonstrate that each molecule of propofol scavenges two radicals, suggesting that 2,6-diisopropylphenol behaves in a manner similar to vitamin E [11], with P· reacting with a further lipid peroxyl radical to form a stable non-radical adduct.

The clinical significance of these observations is not clear. Theoretically, the maximum contribution to plasma antioxidant potential made by propofol is $2x$, where x is the concentration of propofol (μmol litre^{-1}). At a concentration of 20 μg ml^{-1}, equivalent to 112 μmol litre^{-1}, we observed an increase in plasma TRAP of 215 μmol litre^{-1}, which is close to the predicted value of 224 μmol litre^{-1}. (As the plasma protein binding of propofol is quoted as 97–98% [12], it would appear that, in this system at least, total plasma propofol, rather than just free

propofol, is available as an antioxidant.) However, as the anaesthetic concentration of propofol is in the range 2–5 μg ml^{-1} (11–28 μmol $litre^{-1}$) [13], the expected increase in plasma TRAP during propofol anaesthesia would only be in the region of 20–60 μmol $litre^{-1}$, which would not be a significant contribution, given that the normal range of fasting plasma TRAP values is 600–1000 μmol $litre^{-1}$. Nevertheless, as the major lipid-soluble plasma antioxidant is vitamin E, and this comprises only 5–10 % of plasma TRAP (that is, 50–100 μmol $litre^{-1}$) [6], this would represent a doubling of the lipophilic component of the antioxidant capacity of plasma.

Whilst the effect of propofol on plasma antioxidant activity appears to be modest, the similarities between 2,6-diisopropylphenol and vitamin E suggest that attention might be better directed towards its possible effect on tissue antioxidant defences. Vitamin E, present in only small quantities in plasma, plays a crucial role in protecting the lipophilic environment of the cell membrane against oxidant stress [1, 14]. *In vitro*, vitamin E protects endothelial cells [15] and erythrocyte suspensions [11] against free radical-mediated injury. *In vivo*, its deficiency is associated with a variety of acute and chronic conditions [16], although its therapeutic value in the absence of established deficiency is unproven, particularly in the acute situation. This may be a consequence, however, of the prolonged time required for administered vitamin E to increase tissue concentrations [17]. In contrast, propofol accumulates in biomembranes far more readily, so that it may be able to boost the antioxidant defences of tissues and, more specifically, lipophilic membrane environments far more rapidly. Considerable further investigation is required before such a hypothesis can be established.

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