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The assessment of the HO· scavenging action of therapeutic agents

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Abstract: A new method is reported for the assessment of the HO· scavenging action of therapeutic agents. It is based on the photolysis of zinc oxide and has a detection limit of 3.3%. The scavenging order of the compounds tested was *penicillamine* > *rentiapril* > *ascorbic acid* > *cysteine* > *glutathione* > *thiomalic acid* > *N-acetylcysteine* > *myocrysin* > *methionine*. None were as effective as DMSO. It is argued that these compounds can have an *in vivo* protective effect where HO· is produced from oxidant producing cells, thus limiting radical induced damage.

Keywords: *Hydroxyl radical; scavenging; thiols.*

Introduction

The HO· radical is believed to cause cellular damage *in vivo*. One postulated action of thiols such as captopril and glutathione was that they react with the radical and offer a protective effect on the membrane [1–3]. The assay described here provides a simple method for the assessment of the relative efficiencies of compounds in scavenging this type of radical. It was a development of an assay first reported for a fundamental study of the photolysis of zinc oxide. It was used to assess the relative scavenging efficiency of N-acetylcysteine, penicillamine, rentiapril, thiomalic acid, ascorbic acid, myocrysin, methionine, cysteine and glutathione. In the assay HO· radicals were generated by photolysis of zinc oxide and the rate of reaction was followed by the HO· dependant photo-oxidation of methyl orange [4].

Photolysis of a zinc oxide suspension initially produced holes and electrons within the dispersed particles.

$$ZnO + h\nu \rightarrow h^+ + e^-.$$

These species either recombined or migrated to the surface. In the latter process, the holes at the surface reacted to form HO· radicals.

$$h^+ + OH^- \rightarrow HO\cdot.$$

If not otherwise scavenged HO· radicals could undergo recombination reactions to give hydrogen peroxide.

$$2HO\cdot \rightarrow H_2O_2.$$

In addition and almost instantaneously, the electrons produced reduced molecular oxygen.

$$2e^- + O_2 + H_2O \rightarrow HO_2^- + HO^-.$$

The oxidation of methyl orange was used to monitor HO· production. The reaction required two surface adsorbed radicals and involved attack at the azo bond. The dye photofaded, and the loss of colour was monitored spectrophotometrically.

Addition of an effective HO· scavenger [5], reduced the number of available radicals and hence the rate of photodegradation. The difference in absorbance gave a quantitative measure of the scavenging ability of the added compound.

Materials and Methods

All the compounds used were supplied by the Sigma Chemical Co., except N-acetylcysteine which was supplied by Zambon Research.

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A stock solution consisting of 3×10^{-4} M methyl orange and a suspension of zinc oxide equivalent to 0.025 M were prepared in a 5×10^{-3} M sodium borate buffer (pH 9.2). Sample solutions were prepared from the stock in duplicate. The best results were obtained by using 4.2 ml methyl orange solution and 7.5 ml zinc oxide suspension and the volume made up to 22.5 ml with borate buffer. Each scavenger was prepared in borate buffer at a concentration of 10^{-4} M. Of this solution 2.5 ml was added to 22.5 ml of the reaction mixture so that a final concentration of 10^{-5} M was obtained. The blank solutions contained only zinc oxide and methyl orange. A blank was also performed for methyl orange only.

The flask containing the 25 ml solution was placed 10 cm away from a 225 watt light source, and was stirred at constant speed. 3.5 ml aliquots were removed after 0 and 120 min. A second solution was stored in the dark and an aliquot taken at 0 and 120 min. All aliquots were centrifuged at approximately 3,500 r.p.m. for 5 min to remove suspended zinc oxide. 3 ml of each supernatant was removed and placed in a cuvette. UV/Visible spectra were recorded between 200 and 600 nm directly from the cuvette using a Phillips PU8750 spectrophotometer (Fig. 1).

To validate that the reaction was HO· mediated, dimethyl sulphoxide (DMSO) was added to the methyl orange/zinc oxide suspension at concentrations of 10^{-5} M and 10^{-6} M. Aliquots were removed after 0 and 120 min. Spectra were recorded as before.

1000 units of the enzyme catalase were also added to the methyl orange/zinc oxide suspension. Again, aliquots were removed after 0 and 120 min. Spectra were recorded.

By subtracting the baseline at 600 nm from the major methyl orange peak at 465 nm, the % photo-oxidation was calculated for the blank and each of the compounds tested. This was converted into scavenging efficiency by the following equation;

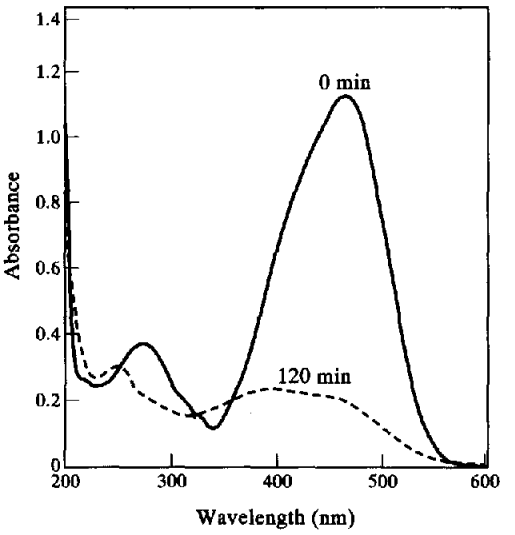

Figure 1
UV-visible absorbance spectra of methyl orange before and after photo-oxidation by zinc oxide.

To determine the standard deviation intrinsic within the experiment, the methyl orange/zinc oxide solution was incubated with glutathione (10^{-5} M). This experiment was performed eight times to attain a value for the standard deviation.

Results and Discussion

This simple assay has potential for the assessment of HO· scavenging. Methyl orange was used as an indicator since it was known to be light stable [4] and a solution of methyl orange with no zinc oxide showed no appreciable photo-oxidation in the assay procedure. Graphs were constructed to evaluate the linear range of the assay and are illustrated in Fig. 2 for glutathione and N-acetylcysteine. The linear range of the assay was compound dependent. In this region the scavenging efficiency increased with the concentration of compound used.

In all cases the sample kept in the dark showed no change in the major methyl orange

$$\text{Scavenging Efficiency \%} = [100 - (\text{\% Photo-oxidation of sample} \times \frac{100}{\text{\%Photo-oxidation of blank}})]$$

where;

$$\text{\% Photo-oxidation} = \frac{(\text{Compound Abs at 465 nm at 0 min}) - (\text{Compound Abs at 465 nm at 120 min})}{(\text{Compound Abs at 465 nm at 0 min}) - (\text{Compound Abs at 600 nm at 0 min})}$$

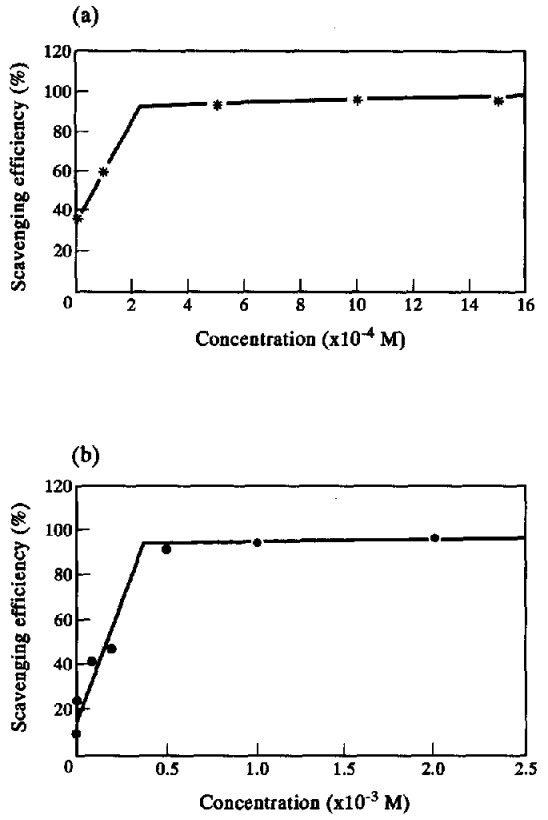

Figure 2
Effect of concentration on scavenging efficiency of (a) glutathione and (b) N-acetylcysteine.

peak at approximately 465 nm, indicating that photolysis is the dominant process. According to the theory outlined earlier, the reaction sequence was HO· mediated. To test this hypothesis, a known HO· scavenger, dimethyl sulphoxide, was added. Photo-oxidation of methyl orange was only significant at concentrations of DMSO lower than 10^{-6} M.

To prove that the reaction was not hydrogen peroxide mediated, 1000 units of the enzyme catalase were added to the solution [16]. The results showed that the photo-oxidation of methyl orange was decreased by approximately 15%, but the dominant process was the photo-oxidation of the methyl orange species. The reduction in signal may have been due to reaction with hydrogen peroxide or to a solvent cage effect, since HO· attack may be hindered due to the steric size and high concentration of the enzyme catalase.

The detection limit was calculated from the standard deviation obtained after the scavenging action of glutathione at 10^{-5} M was determined (n = 8). The detection limit was taken as three times the standard deviation and was calculated to be 3.3%.

To confirm that the changes were not merely due to changes in the pH of the solution during photolysis, pH was monitored for each aliquot taken. The pH remained constant at 9.2 throughout.

The results obtained are shown in Table 1. They show that penicillamine was the most effective scavenger, and methionine the least effective. The methionine result was significant since it showed that compounds which are not HO· radical scavengers do not interfere with the assay.

Table 1
Scavenging efficiency of various biologically important compounds

Compound at 10^{-5} M	Scavenging efficiency (%)
Dimethyl sulphoxide	96
Penicillamine	60.0
Rentiapril	48.8
Ascorbic acid	37.9
Cysteine	37.3
Glutathione	28.0
Thiomalic acid	27.5
N-Acetylcysteine	23.1
Myocrysin	13.8
Methionine	1.3

The pathogenesis of radical induced damage in disease is only partially understood. At cell sites where processes such as phagocytosis occur, the radical burst produces a range of radicals in concentrations unlikely to be affected by scavenging efficiencies of the order found here. The highly active HO· radicals, if formed, will attack naturally occurring sites such as double bonds. The only likely direct action of these compounds studied is to limit the radius of the damage by scavenging the reducing HO· concentrations away from the site of production. This does not apply to the same extent with glutathione where the natural concentration in some cells is relatively high (approx 2 mM in the erythrocyte). However, the sites of damage are often remote from the site of radical production. For damage to occur at remote sites, a carrier species such as hypochlorite or peroxide is required with the active radical being produced *in situ*, possibly by reaction with iron (III) [7, 8]. In these cases, concentrations of HO· will be low but effective.

In such circumstances the level of scavenging may be sufficient for almost complete removal

of HO· and it may be the key factor in preventing reactions at a specific site [9]. The assay developed here provides a simple method to test the scavenging efficiency of both exogenous and endogenous compounds.

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