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THE NEUTRALIZATION OF HYDROXYL RADICAL BY SILIBIN, SORBINIL AND BENDAZAC

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The compounds silibin, sorbinil and bendazac act as hydroxyl radical scavengers, when the hydroxyl radical is generated by the Fenton reaction. Hydroxyl was detected by the degradation of deoxyribose.

The Authors discuss the possibility that the scavenger activities of those compounds may explain, at least in part, their therapeutic activity.

KEY WORDS: hydroxyl, sorbinil, bendazac, silibin.

INTRODUCTION

Free radicals may be generated during normal metabolic processes, after exposure to ionizing radiations or through the action of xenobiotics.[1–4] If formed in the cell they affect all its constituents, namely membranes, DNA, proteins and proteoglycans. These damaging effects may be limited by the presence of elevated concentrations of free radical scavengers, provided that radicals formed from the scavengers are not damaging themselves.[6]

It is of interest to study the capacity of some drugs as free radical scavengers in order to understand better the mechanism of their protective effect. In the present work we studied the capacity of silibin, sorbinil and bendazac to scavenge hydroxyl radicals, generated in the reactions of Fenton and of Haber–Weiss.[7]

Silibin is a flavonoid extracted from the milk thistle.[8] It inhibits lipid peroxidation and GSH depletion induced in rat liver by acute ethanol intoxication and is used in the treatment of liver diseases, especially in the intoxication by mushrooms containing amanitin and phalloidin.[9] It is also a potent inhibitor of cyclic AMP phosphodiesterase[10] and favors platelet aggregation.[11] It has also been reported as a hepatoprotector against D-galactosamine intoxication in the rat.[12] On isolated liver cells it protects against lysis by hypotonic solutions.[13]

Sorbinil is a derivative of hydantoin (spirooxazolidinedione). It is a powerful inhibitor of aldose reductase, preventing accumulation of sorbitol in the sciatic nerve of diabetic rats, reducing its concentration in the retinal capillaries and in the lens and decreasing proteinuria in diabetic patients.[14,15]

Bendazac is 1-benzyl-indazole-3-oxyacetic acid. It is used as a treatment against senile cataract, since it protects the lens from denaturation, aggregation and precipitation.[16] It also has antinflammatory properties.[17]

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MATERIAL AND METHODS

Reagents

All reagents were obtained in the maximum degree of purity available and used as supplied. Deoxyribose, sodium salicylate and xanthine oxidase were obtained from Sigma; ammonium ferrous sulphate from "May and Baker"; sodium formate from "Carlo Erba" and sodium benzoate from Bush. Silibin, sorbinil and bendazac were supplyed by Laboratories "Madaus", "Pfizer" and "Lepori", respectively.

The remaining reagents were obtained from "Merck".

STUDY OF HYDROXYL SCAVENGERS

1) Generation of hydroxyl radicals by a mixture of ferrous salts and hydrogen peroxide. Detection by deoxyribose degradation with formation of thiobarbituric acid products[18]

0.2 ml of 0.15 M NaCl, pH 7.4, together with 0.2 ml of deoxyribose, 5 mM, were added to clean glass tubes. 0.2 ml of the appropriate radical scavengers was added followed by 0.1 ml H_2O_2 0.96 mM and 0.1 ml of ammonium ferrous sulphate 0.96 mM. When EDTA was added, it was added in first place (0.1 ml EDTA 1.44 mM) followed by the ammonium ferrous sulphate. The scavengers did not affect the pH of the reaction mixture, which was practically constant during the experiments.

The volume of each tube was made to 0.8 with distilled deionized water, and the samples incubated at 37°C for 1 hour. Following incubation TBA reactivity was developed by heating for 15 min at 100°C, after adding 0.5 ml 1% TBA in 0.05 M Na OH and 0.5 ml 2.8% (w/v) trichloroacetic acid. The resulting chromogens were measured at A_{532nm} against appropriate blanks.

2) Formation of OH· by hypoxanthine/xanthine oxidase in the presence of iron salts. Detection by hydroxylation of salicylate.

The quantity of dihydroxylated product formed may be decreased in the presence of OH· scavengers. This assay was executed following the technique described by Richmond *et al.*[14]

In the final part of execution of both methods, after acidification with concentrated HCl and TCA, respectively in the methods for salicylate and deoxyribose, the scavengers (silibin, sorbinil and bendazac) precipitate, but this fact does not interfere, only requires a centrifugation to discard the precipitates.

RESULTS

The results obtained with the system containing xanthine/xanthine oxidase and iron had to be discarded, since we found that all 3 compounds tested are strong inhibitors of the enzyme. Under these circumstances it was impossible to ascertain if the decreased production of OH· is due to the scavenger properties or to a lowered activity of xanthine oxidase.

TABLE I
Effect of silibin as an OH· scavenger, generated by the Fenton reaction and detected by deoxyribose degradation

	TBA Reactivity	
	A_{532nm}	% inhibition
complete system (c.s.)	0.809	
c.s. + silibin 20 mM*	0.070	91
c.s. + silibin 10 mM	0.085	89
c.s. + silibin 5 mM	0.101	87
c.s. + silibin 1 mM	0.135	83
c.s. + silibin 0.5 mM	0.201	75

The compounds may possibly find a clinical application in the prevention or treatment of reperfusion injury.[21] Further studies on this subject are being pursued.

(a) Silibin (Table I): in the method of deoxyribose the percentage of inhibition goes up to 89% for a concentration of 10 mM. It must be noted that in the assay with silibin a yellowish chromogen is formed after addition of iron. This chromogen does not appear if iron is chelated with EDTA 1.4 mM, but the final results are the same.

(b) Sorbinil: is scarcely soluble in water, but in the concentrations of 1 and 0.5 mM it has an inhibitory effect, although moderate, as seen in Table II. When sorbinil is left undissolved up to 10 mM an increasing effect is observed.

(c) Bendazac: the results presented in Table III demonstrate that bendazac is a good OH· scavenger, and the scavenger capacity varies with the concentration of the drug.

(d) Comparative study of the scavenger capacity of the 3 compounds — effect of EDTA: in Table IV we present the comparative study of the 3 scavengers in the same experiment.

Another well-known scavenger, formate, was also assayed for comparison. The values represent the results of one experiment chosen from several experiments performed.

It is observed that in the absence of EDTA, silibin is the strongest OH· scavenger while the other compounds show the same scavenging capacity. In the presence of 0.18 mM EDTA silibin maintains it scavenging power, but the other compounds show an increased capacity.

TABLE II
Effect of sorbinil as an OH· scavenger, generated by the Fenton reaction and detected by deoxyribose degradation

	TBA Reactivity	
	A_{532nm}	% inhibition
complete system (c.s.)	0.809	
c.s. + sorbinil 10 mM	0.342	58
c.s. + sorbinil 5 mM	0.365	55
c.s. + sorbinil 1 mM	0.495	39
c.s. + sorbinil 0.5 mM	0.605	25

*In this and in the other tables we refer to final concentrations of the scavengers in the reaction mixtures.

TABLE III
Effect of bendazac as an OH· scavenger, generated by the Fenton reaction and detected by deoxyribose degradation

	TBA Reactivity	
	A_{532nm}	% inhibition
complete system (c.s.)	0.806	
c.s. + bendazac 20 mM	0.160	80
c.s. + bendazac 10 mM	0.272	66
c.s. + bendazac 5 mM	0.345	57
c.s. + bendazac 1 mM	0.352	56
c.s. + bendazac 0.5 mM	0.492	39

A possible explanation might be that in the absence of EDTA iron salts are bound to the scavengers; if this happens a part of these scavengers would be unable to react with OH·.[20]

DISCUSSION

We demonstrated that 3 compounds, silibin, sorbinil and bendazac, possess antioxidant properties in vitro, as scavengers of hydroxyl radical generated by the Fenton reaction.

Silibin proved to be the strongest scavenger and seems to have iron binding properties, since its activity is the same in the presence and in the absence of EDTA.[20]

The fact that these compounds possess antioxidant properties may contribute to explain the effect of bendazac and of sorbinil in the treatment of cataract and of silibin as a protector of liver parenchyma. But we must remember that although a drug is an active OH· scavenger in simplified systems *in vitro*, other factors may interfere with its effects *in vivo*. These factors include toxicity, short half life in biological systems and non ideal tissue distribution.

The scavenger to be effective in a biological system must be in the right location, in an appropriate concentration, and both the scavenger and its reaction product must have an acceptable toxicity.

TABLE IV
Comparison of silibin, sorbinil, bendazac and formate as scavengers of OH·, generated by the Fenton reaction and detected by the degradation of deoxyribose, in the absence and in the presence of EDTA 0.18 mM

	TBA Reactivity	
	A_{532nm}	inhibition
complete system (c.s.)	0.794	–
c.s. + silibin 10 mM	0.081	90
c.s. + sorbinil 10 mM	0.316	60
c.s. + bendazac 10 mM	0.294	63
c.s. + formate 10 mM	0.269	66
c.s. + EDTA 0.18 mM	0.915	–
c.s. + EDTA + silibin 10 mM	0.070	92
c.s. + EDTA + sorbinil 10 mM	0.044	95
c.s. + EDTA + bendazac 10 mM	0.048	94
c.s. + EDTA + formate 10 mM	0.174	81

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