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THE EFFICACY OF MONOHYDROXAMATES AS FREE RADICAL SCAVENGING AGENTS COMPARED WITH DI- AND TRIHYDROXAMATES

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Abstract—Desferrioxamine, the therapeutic iron chelator, is limited in its usage by its short half-life in plasma and lack of oral activity, its side-effects and its slow penetration into cells. Several studies have emerged recently demonstrating the ability of this trihydroxamate compound to act as a radical scavenger, in addition to and independently of its iron-chelating properties. These include the interaction of desferrioxamine with the superoxide radical and ferryl myoglobin radical, as well as its action as a chain-breaking antioxidant in peroxidizing erythrocyte membranes. We have synthesized recently a series of monohydroxamate compounds and investigated their efficacy as radical scavenging antioxidants in comparison with desferrioxamine and rhodotorulic acid, a naturally occurring dihydroxamate compound. The results show that the relative rates of reaction of these hydroxamate derivatives with ferryl myoglobin are *N*-methyl-*N*-hexanoyl hydroxylamine>*N*-methyl-*N*-benzoyl hydroxylamine>*N*-methyl-*N*-acetyl hydroxylamine>desferrioxamine>rhodotorulic acid>*N*-methyl-*N*-butyryl hydroxylamine.

Desferrioxamine is a trihydroxamate hexadentate iron chelator applied clinically for the treatment of iron overload. The hexadentate nature of the ligand allows chelation of iron III ions on an equimolar basis, with a binding constant of 10^{31} [1]. This high binding constant together with the redox properties of the complex make it a very poor catalyst for free radical production [2]. Many studies have emphasized the action of desferrioxamine in protecting cells and tissues from free radical-induced damage, for example in the pathogenesis of myocardial tissue destruction during reperfusion injury [3–5].

A recent report of the beneficial effects of desferrioxamine administration in a canine model of myocardial reperfusion injury [4] has been interpreted as implicating the involvement of chelatable iron in the pathogenesis of reperfusion injury; once iron has been bound by desferrioxamine, it is no longer considered able to catalyse processes such as hydroxyl radical formation. Shuter *et al.* [5] have directly demonstrated, by use of EPR spectroscopy, the inhibition of radical production by desferrioxamine during reperfusion of isolated rat hearts, implicating a role for iron catalysis in radical production. Several studies have demonstrated recently that desferrioxamine has an activity other than as a metal chelator [6–11].

Recent work has proposed that the trihydroxamate moiety could be involved as a protective agent through its ability to donate hydrogen atoms or electrons to a variety of systems including ferryl myoglobin [9, 12], ferryl haemoglobin [13], activated horseradish peroxidase [7] and activated cytochromes [8], as well as the superoxide radical [6]. In addition, the ability of desferrioxamine to intercept the propagation phase of membrane peroxidation has also been demonstrated [9–11] with the formation of the desferrioxamine nitroxide radical [11]. The results of Reddy *et al.* [4] highlight the relatively limited therapeutic efficacy of desferrioxamine in attenuating post-ischaemic contractile dysfunction through its inability to reduce infarct size. The ability of desferrioxamine to enter, on a physiologically relevant timescale [14], only those cells undergoing active pinocytosis [15] may explain this.

In this study we have synthesized a range of monohydroxamate compounds and investigated their efficacy as free radical scavenging agents compared to the hexadentate trihydroxamate desferrioxamine and the bidentate dihydroxamate rhodotorulic acid (Fig. 1). These compounds have been investigated as antioxidants against activated haem protein species, lipid peroxyl radicals in peroxidizing membranes and against radicals generated during deoxyribose degradation.

MATERIALS AND METHODS

Myoglobin (ferric form, horse heart, type III), Sephadex G-15, bovine serum albumin (98–99% albumin), Folin and Ciocalteu's phenol reagent, thiobarbituric acid and malonaldehyde bis-(dimethylacetal), 1,1,3,3-methoxypropane derivative, were all purchased from the Sigma Chemical Co. (Poole, U.K.). Desferrioxamine mesylate

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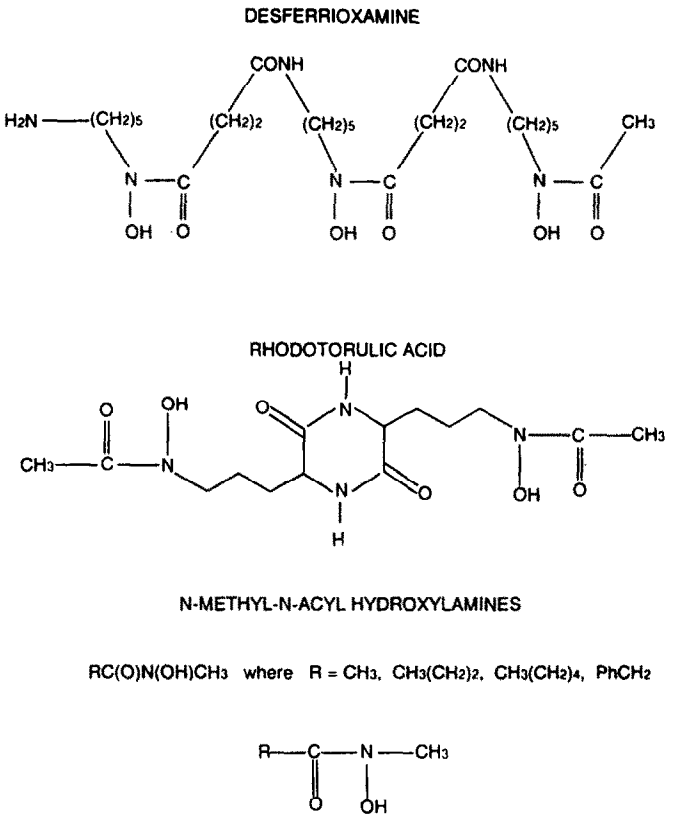


Fig. 1. Structures of hydroxamate compounds: desferrioxamine; rhodotorulic acid; *N*-methyl-*N*-acyl hydroxylamines: R=CH_3 (NMAH*), R=$CH_3(CH_2)_2$ (NMBH), R=$CH_3(CH_2)_4$ (NMHH), R=$PhCH_2$ (NMBzH).

(desferal) and rhodotorulic acid were from CIBA-Geigy; all other hydroxamates (alternatively named as *N*-methyl *N*-acyl hydroxylamines or *N*-methyl *N*-hydroxyamides), $RC(O)N(OH)CH_3$ with R=CH_3, $CH_3CH_2CH_2$, $CH_3(CH_2)_4$, $PhCH_2$, were synthesized in the Department of Chemistry, University of York, using standard literature methods [16]. Hydrogen peroxide was of Aristar grade and all other chemicals were of Analar grade and supplied by Merck (Darmstadt, Germany). Metmyoglobin was prepared as described previously [17] and purified by oxidation with potassium ferricyanide and subsequent separation on a Sephadex G-15 column. Visible spectroscopy was performed on a Beckman DU-65 spectrophotometer fitted with Quant 1 software and linked to an IBM PC/2. Incubations of metmyoglobin (final concentration 20 μM) were carried out in 5 mM phosphate-buffered saline, pH 7.4 and spectra were run at timed intervals for up to 1 hr. The concentration of the different myoglobin species were estimated using the Whitburn algorithms based on the extinction coefficients for ferryl, met- and oxymyoglobin [18]. Rate constants for the formation and decay of ferryl myoglobin in the presence of the hydroxamate compounds were calculated applying a least squares fitting programme. The radical scavenging activities of these compounds were assessed by use of the deoxyribose assay [19]. Iron was used in the assay as ferrous ammonium sulphate (100 μM), deoxyribose at a concentration of 2 mM and the drugs at final concentrations 0–300 μM and the duration of incubation was 1 hr.

Human haemoglobin-free erythrocyte membranes were prepared from normal fresh erythrocytes in phosphate-buffered saline at pH 7.4 according to the procedure of Dodge *et al.* [20]. Membrane concentrations were assessed by assaying protein by the method of Lowry *et al.* [21]; a final concentration of 1 mg protein/mL was normally employed. The effect of the hydroxamate compounds on peroxidation of the membranes induced by activated myoglobin was assessed by applying the thiobarbituric acid assay [22]. Appropriate controls were applied according to Gutteridge [23]. The absorbance of the chromophore was measured at 532 nm and the background absorbance at 580 nm due to possible contributions from the haem protein absorption was subtracted. Standards were run simultaneously under the same conditions using malondialdehyde prepared by acid hydrolysis of the acetal.

Free radical generation was monitored directly

* NMAH, *N*-methyl-*N*-acetyl hydroxylamine; NMHH, *N*-methyl-*N*-hexanoyl hydroxylamine; NMBH, *N*-methyl-*N*-butyryl hydroxylamine; NMBzH, *N*-methyl-*N*-benzoyl hydroxylamine.

using a Bruker ESP300 EPR spectrometer equipped with 100 kHz modulation and a Bruker ERO35M gaussmeter for field calibration. Hyperfine coupling constants were measured directly from the field scan. Signal intensities (which are directly proportional to the radical concentration for a given species) were determined by measurement of peak to peak line heights on spectra recorded with the use of identical spectrometer settings. All values are the means of at least three separate determinations.

RESULTS

Spectroscopic studies

Activation of metmyoglobin by hydrogen peroxide (1:1.25 molar ratio) induces a rapid development of the spectral characteristics attributable to ferryl myoglobin (peaks at 515, 550 and 585 nm) with the concomitant loss of the metmyoglobin peak at 630 nm. Application of the Whitburn algorithms, which allow the relative proportions of ferryl, met and oxy to be calculated, indicates that the concentration of ferryl myoglobin reaches a maximum value at 10 min (Fig. 2A), at which time it constitutes *ca.* 60% of the total haem [12] and remains at approximately this level for 60 min.

The monohydroxamate compounds (*N*-methyl-*N*-acetylhydroxylamine, NMAH; *N*-methyl-*N*-hexanoylhydroxylamine, NMHH; *N*-methyl-*N*-butyrylhydroxylamine, NMBH; *N*-methyl-*N*-benzoylhydroxylamine, NMBzH) have been compared to desferrioxamine and rhodotorulic acid (a trihydroxamate and a dihydroxamate, respectively), in terms of their suppressive effects on the oxidative activation of the haem protein, using two experimental approaches: firstly, addition of the activating agent (H_2O_2) to the myoglobin after pretreatment with the compound (Fig. 2A), the latter thus being present during the formation of the ferryl myoglobin species; and secondly, addition of the hydroxamate to pre-formed ferryl myoglobin (Fig. 2B). The spectra indicate the effects of the different agents at a range of concentrations on the formation and suppression of ferryl myoglobin.

When reaction mixtures containing desferrioxamine (added prior to the addition of H_2O_2) were investigated, the maximum concentration of ferryl myoglobin was found to depend on the concentration of desferrioxamine present. At levels of 50 and 100 μM, i.e. in excess of the myoglobin concentration, the maximal level (50%) peaked at $t = 3$ min and the ferryl species was totally reduced back to metmyoglobin by 25 min. At 25 μM desferrioxamine, there was less suppression of the maximal concentration of ferryl myoglobin and this level was attained at slightly earlier times than in the absence of the compound; at this concentration reduction back to the met form was complete by 45 min. With 10 μM desferrioxamine, enhanced ferryl myoglobin formation was initially observed, after which reduction by the hydroxamate was apparent. This enhancement of ferryl myoglobin formation also occurred with the other hydroxamates tested with rhodotorulic acid, NMAH and NMBzH showing more pronounced effects than desferrioxamine.

The rate constants for the reaction of ferryl myoglobin with the antioxidant compounds were determined. To model the data we have adopted the equation:

$$N = \frac{\lambda_1}{\lambda_2 - \lambda_1}(e^{-\lambda_1 t} - e^{-\lambda_2 t}).$$

In this context we attach no physical significance to the constant λ_1, but we take λ_2 as a fair description of the exponential decay of the efficacy of the drug. The equation has been fitted to the data with a least squares minimization programme. A typical representation is shown in Fig. 3 and the data in Table 1. The data show that the hydroxamates react with ferryl myoglobin in the order shown: NMHH (110 M^{-1} sec^{-1}), NMBzH (66 M^{-1} sec^{-1}), NMAH (50 M^{-1} sec^{-1}), desferrioxamine (38 M^{-1} sec^{-1}), rhodotorulic acid (30 M^{-1} sec^{-1}), NMBH (27 M^{-1} sec^{-1}), suggesting that their reactivity and efficacy in reducing ferryl myoglobin to metmyoglobin is independent of the number of hydroxamate groups in the molecule.

Addition of desferrioxamine to the pre-formed ferryl myoglobin at $t = 15$ min induced progressive reduction of ferryl myoglobin (Fig 2B), with 50 and 100 μM causing total suppression 45 min after addition, and 25 μM and 10 μM inducing 92% and 85% reduction respectively, at this time.

EPR studies

Examination of the above reaction mixtures by EPR spectroscopy with the compounds present at either 50 or 100 μM, resulted in the detection of signals which could be assigned to the corresponding nitroxide radicals formed by oxidation of the N-OH group to N-O$^{\cdot}$ as the ferryl myoglobin is reduced to the met form. Thus, desferrioxamine gives rise to signals from the desferrioxamine nitroxide radical (with hyperfine coupling constants as determined previously [6]). NMAH, NMBH, NMHH and NMBzH produced signals which were consistent with the presence of radicals with partial structure $CH_3N(O^{\cdot})C(O)$- (with parameters a_N 0.780, a_{3H} 0.886 mT), and rhodotorulic acid gave signals assignable to a radical with partial structure $CH_2N(O^{\cdot})C(O)$- (a_N 0.767, a_{2H} 0.661 mT) (Fig. 4) as would be expected for the nitroxide radicals produced from the parent compounds.

Table 1. Rate constants determined for the reaction of 50 μM hydroxamate

Compound	λ_2	χ^2	Rate constant ($M^{-1}sec^{-1}$)
Desferrioxamine	0.1150	4.1	38
Rhodotorulic acid	0.0892	2.5	30
NMAH	0.1530	3.1	50
NMBH	0.0801	3.3	27
NMBzH	0.1971	2.5	66
NMHH	0.3295	2.4	110

χ^2 is an expression of the goodness of fit of the experimentally observed points to the calculated line.

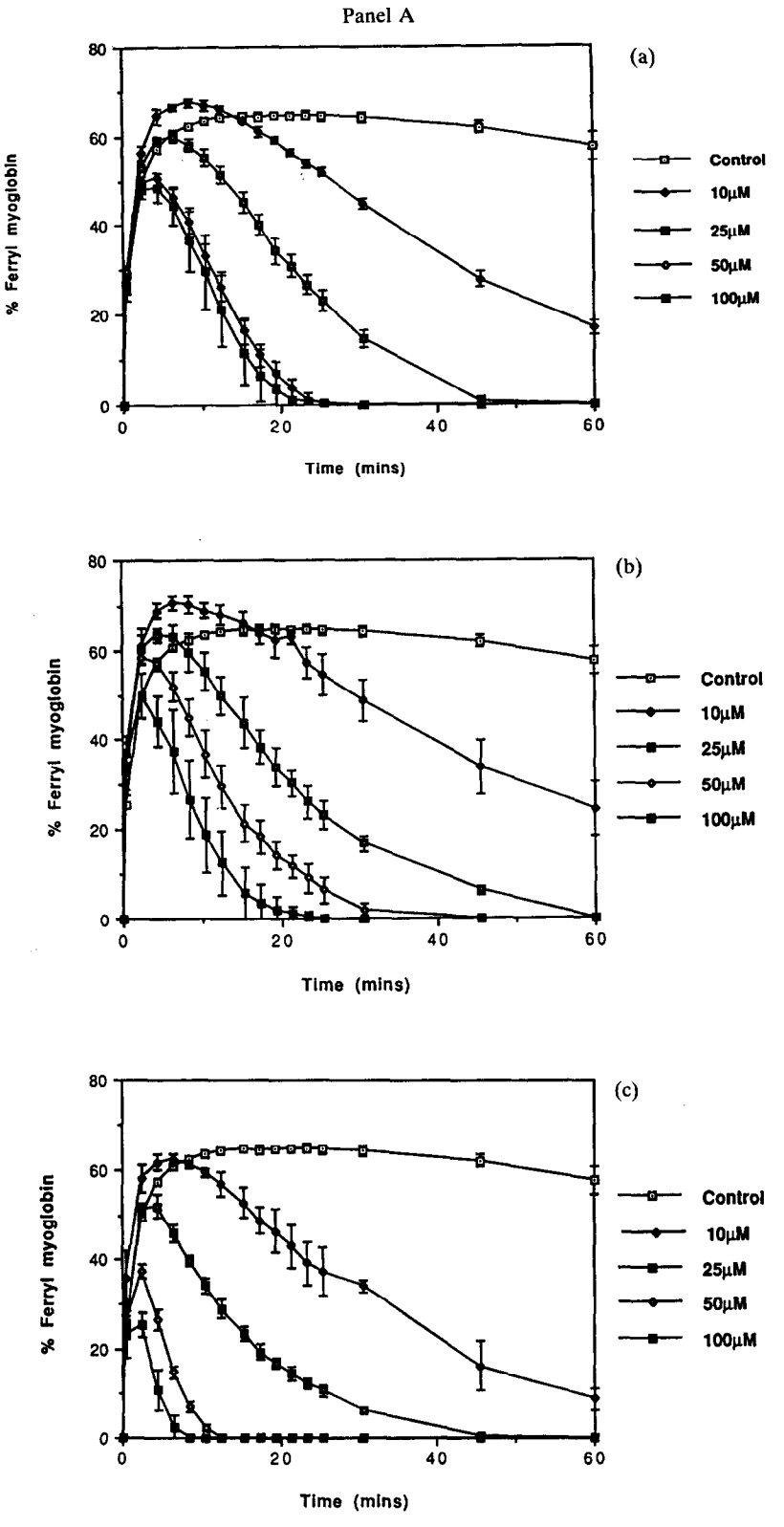
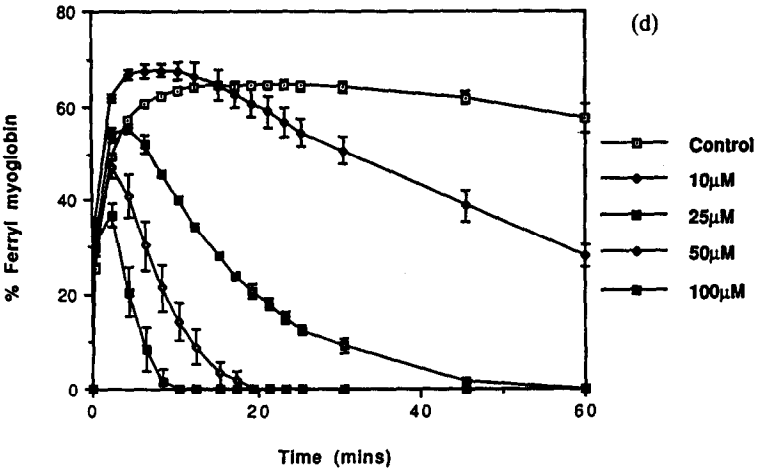
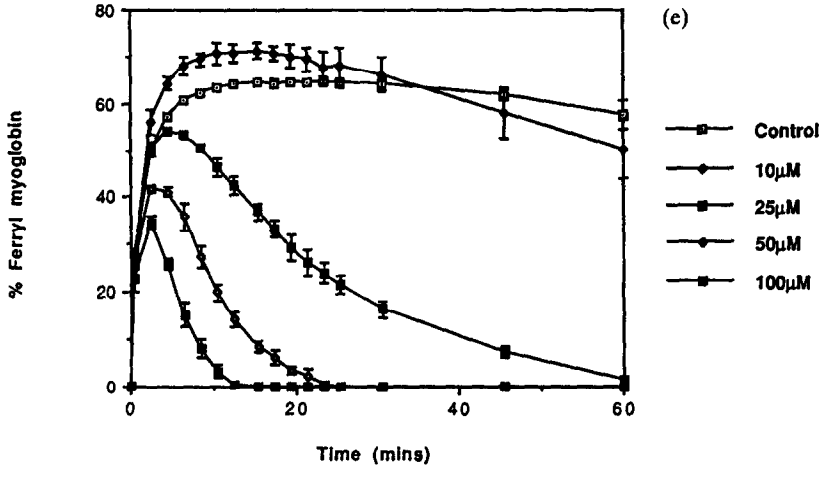
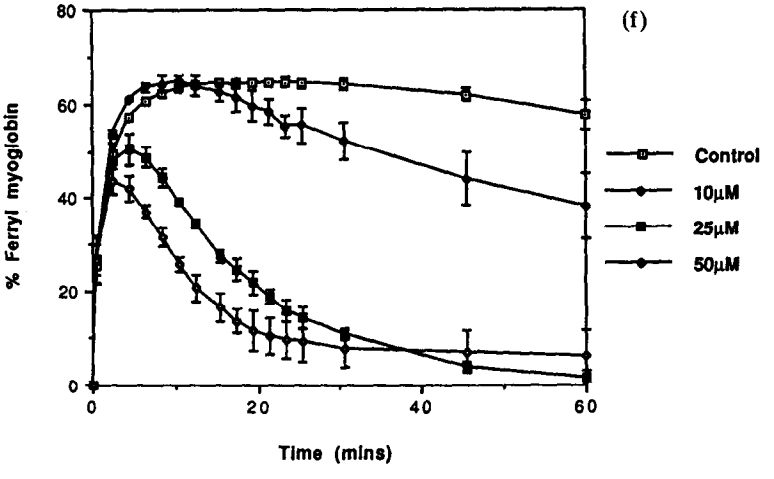


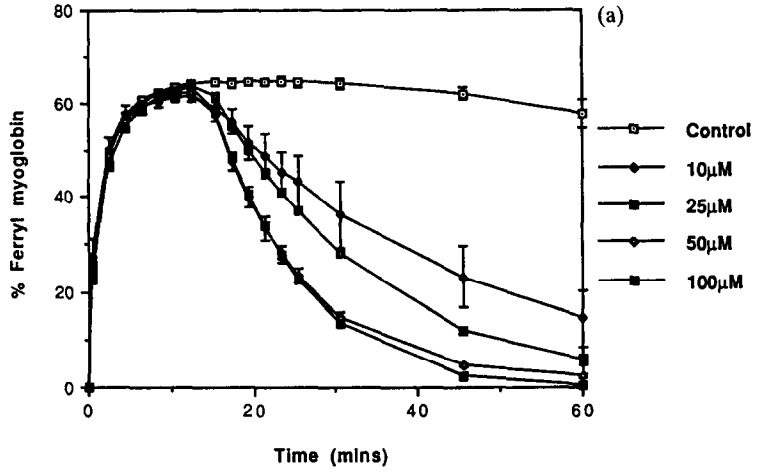
Fig. 2. The effects of the hydroxamate compounds on ferryl myoglobin formation as a function of time. Panel A: drugs added prior to peroxide activation; panel B: drugs added 15 min after peroxide addition. [Metmyoglobin (20 μM), hydrogen peroxide (25 μM), phosphate buffer pH 7.4]. (a) Desferrioxamine; (b) rhodotorulic acid; (c) NMHH; (d) NMBzH; (e) NMAH; (f) NMBH. Error bars ± SD, N=3.

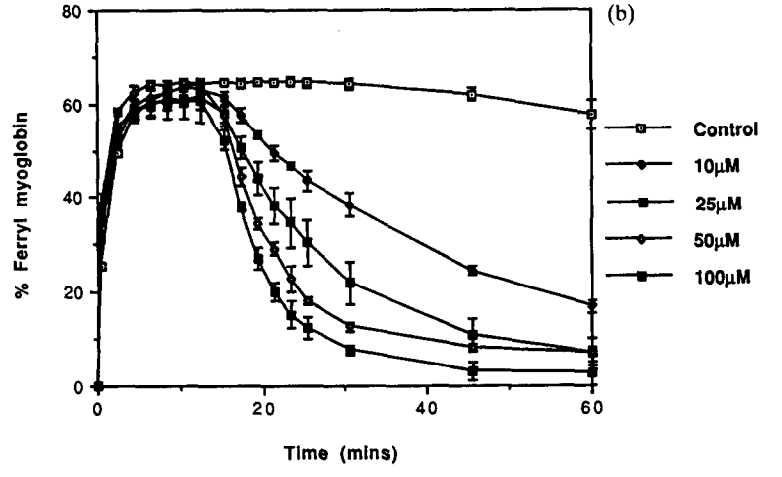

(d)
% Ferryl myoglobin
Time (mins)
Control
10μM
25μM
50μM
100μM

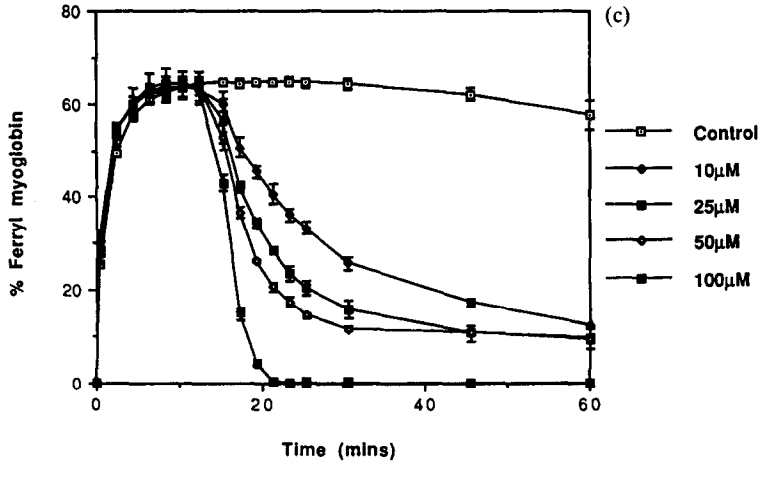

(e)
% Ferryl myoglobin
Time (mins)
Control
10μM
25μM
50μM
100μM

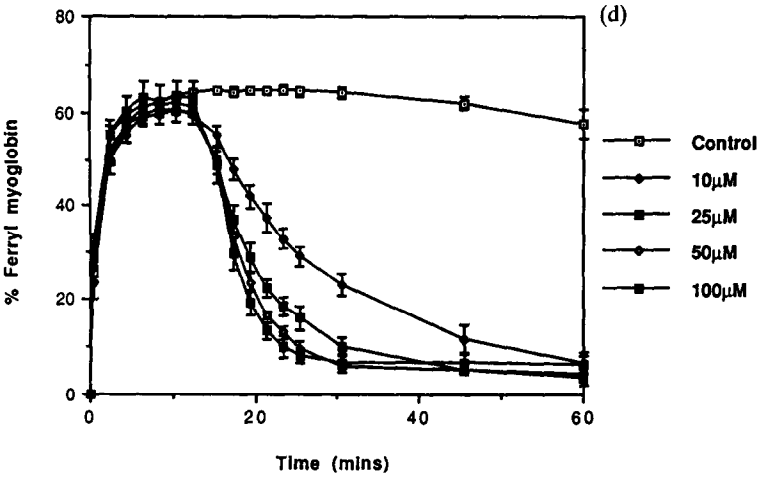

(f)
% Ferryl myoglobin
Time (mins)
Control
10μM
25μM
50μM

Panel B


(a)
% Ferryl myoglobin
Time (mins)
Control
10μM
25μM
50μM
100μM


(b)
% Ferryl myoglobin
Time (mins)
Control
10μM
25μM
50μM
100μM


(c)
% Ferryl myoglobin
Time (mins)
Control
10μM
25μM
50μM
100μM



Inhibition of 2-deoxyribose degradation

The reactivity of the hydroxamate compounds against radicals capable of inducing the degradation of deoxyribose is shown in Fig. 5. The data show that 50 μM NMAH and desferrioxamine were equally effective in inhibiting radical-induced degradation, whereas NMHH and NMBH were ineffective. As the concentration increased, desferrioxamine was increasingly and proportionately more effective whereas this was not the case with NMAH.

Membrane lipid peroxidation studies

Activation of metmyoglobin (20 μM) by hydrogen peroxide (1.25-fold molar excess) in the presence of haemoglobin-free erythrocyte membranes (0.5 mg of membrane protein/mL) induced a significant stimulation of lipid peroxidation at 3 hr, to 0.69 ± 0.1 nmol/mg of protein compared to controls as reported previously [9, 12]. Addition of desferrioxamine at 10 or 50 μM (at the time of myoglobin activation in the presence of membranes) resulted in 60 and 74% inhibition, respectively. NMAH at 10 μM was less efficacious (36% inhibition) than desferrioxamine, whereas at 50 μM it was as effective. The explanation for this difference may lie in the fact that, when the compound is present *prior* to activation of the metmyoglobin and the initiation of peroxidation, the hydroxamate can act in two ways: firstly, inhibiting the formation of the ferryl myoglobin species; and secondly, by acting as a chain-breaking antioxidant by reacting with lipid peroxyl radicals. Desferrioxamine reacts more rapidly with the initiating species than the monohydroxamates. When the compounds are added to the pre-formed ferryl myoglobin 1½ hr after initiating lipid peroxidation and the peroxidation continued for a further 1½ hr, the action of the hydroxamate will be essentially to suppress any further lipid peroxidation. Under these conditions 10 μM NMAH was as effective as 10 μM desferrioxamine (*ca.* 20%, protection) whereas 10 μM rhodotorulic acid was less active. However, at 50 μM, all three compounds are equally efficacious.

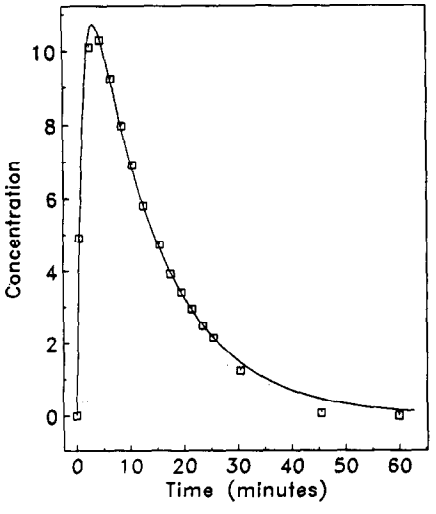


Fig. 3. Plot of ferryl myoglobin concentration (μM) as a function of time in the presence of 25 μM NMHH added prior to activation. Typical least squares fit for assessing rate constants of the reaction of ferryl myoglobin with NMHH.

DISCUSSION

Desferrioxamine, the hexadentate iron chelator, has been compared with its hydroxamic acid analogues, rhodotorulic acid and a number of *N*-methyl monohydroxamate derivatives with varying substituents (acetyl-, butyryl-, benzoyl- and hexanoyl-) for radical scavenging potential. All these compounds are iron chelators, desferrioxamine and rhodotorulic acid promoting iron excretion from iron-loaded rats *in vivo* [24–26]. Of the monohydroxamates applied here only *N*-methyl acetohydroxamic acid has been evaluated as an iron chelator in *in vivo* animal models; NMAH, while having a high and selective affinity for iron (with $k = 10^{28}$), showed only a low tendency for depleting liver iron [27]. Hydroxamates have also been

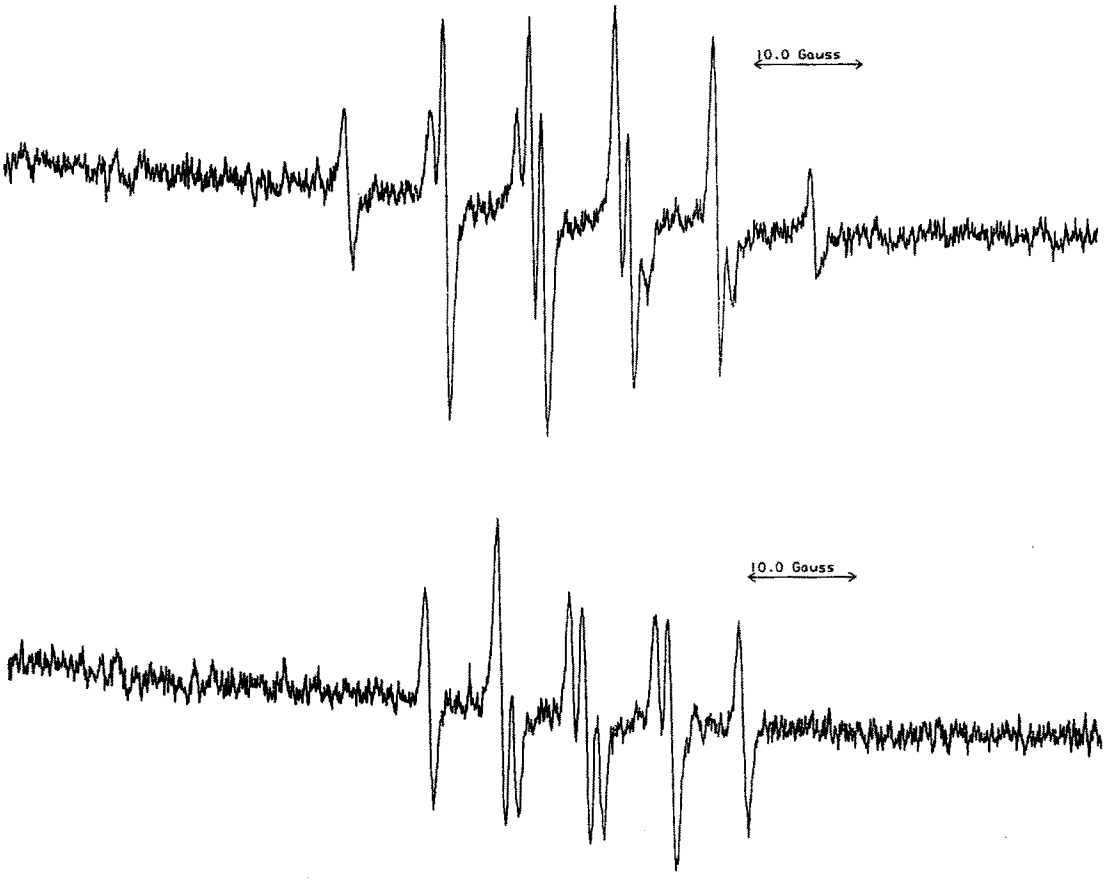


Fig. 4. EPR spectrum of the nitroxide radicals derived from NMAH (100 μM) (upper) and rhodotorulic acid (50 μM) (lower) on reaction with metmyoglobin (20 μM) activated with hydrogen peroxide (25 μM) in 5 mM phosphate buffer, pH 7.4. For analysis of spectrum and hyperfine coupling constants see text. 10 Gauss = 1 mT.

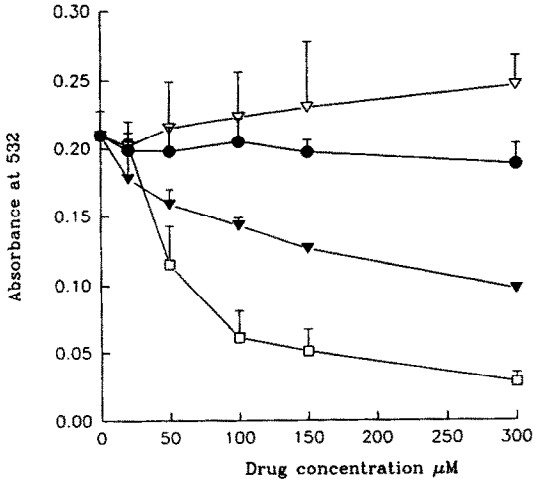


Fig. 5. The effects of the concentration of hydroxamate compounds on deoxyribose degradation assessed as the absorbance at 532 nm of thiobarbituric acid-reactive products. (▽) NMBH, (●) NMHH, (▼) NMAH, (□) desferrioxamine. Error bars ± SD, N=5.

shown to inhibit ribonucleotide reductase at high concentrations, and to inhibit lipoxygenases, presumably by binding to the iron [28, 29] or acting as reducing agents [30].

A number of reports over the last 5 years have demonstrated that desferrioxamine has a mechanism of action other than as an iron chelator [6–10], namely that of a free radical scavenger. The hydroxamate moieties of desferrioxamine, the groups involved in the binding of iron, can act as reducing agents through their ability to donate hydrogen atoms or electrons with the consequent formation of the desferrioxamine nitroxide radical. Desferrioxamine has been shown to be effective in a variety of systems involving haem proteins activated by hydrogen peroxide such as ferryl myoglobin [8, 9], ferryl haemoglobin (Rice-Evans, 1992, unpublished observations) and activated horseradish peroxidase [7], as well as the superoxide radical [2, 6, 31]. The EPR spectrum of the desferrioxamine nitroxide radical has been cited as evidence [31–34, 12]. In addition, the ability of desferrioxamine to intercept the propagation phase of peroxidizing membrane

lipids of the erythrocyte [9], the sickle erythrocyte [11] and of model liposomal systems [10] has been demonstrated with the formation of the desferrioxamine nitroxide radical [11]. The formation of similar species when the hydroxamates studied here interact with ferryl myoglobin would suggest a similar mechanism of action.

We have shown previously that the interaction of ferryl myoglobin with a variety of radical scavengers such as ascorbate, Trolox C and salicylate leads to reduction to metmyoglobin by a radical scavenging mechanism and that compounds such as 2-deoxyribose, mannitol and phenylalanine have no effect [35–37, 9].

The observed effects that the drugs studied here have on radical-mediated deoxyribose degradation may relate to the differential redox properties of the complexes with iron as well as their radical scavenging ability. The lack of response of NMBH and NMHH, the two derivatives with longer alkyl chains attached to the carbonyl groups, may be due to these factors or involve steric crowding in the formation of their complexes with iron with the result that these complexes may allow redox cycling of the iron. Their rate constants for scavenging radicals would not be expected to be dramatically different.

Our studies further confirm the action of the monohydroxamates as scavengers of ferryl haem protein radicals as a result of their action as hydrogen atom donors, reducing ferryl myoglobin radical to the met form of the haem protein. The rate constants for this reaction suggest that the reactivity and efficacy of the drugs are independent of the number of hydroxamate groups in the molecule. In addition, it is possible to define a hierarchy of activity of the drugs from these rate constants showing that the NMHH derivative is 3–4 times more efficacious than desferrioxamine; this increase in activity is even more dramatic when calculated on a per mole of hydroxamate basis. These compounds also show considerable activity as chain-breaking antioxidants.

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