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LINEAR AND NON LINEAR COMPETITION PLOTS IN THE DEOXYRIBOSE ASSAY FOR DETERMINATION OF RATE CONSTANTS FOR REACTION OF NON STEROIDAL ANTIINFLAMMATORY DRUGS WITH HYDROXYL RADICALS

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Performing the deoxyribose (DR) assay for determination of the rate constants for reaction of non steroidal antiinflammatory drugs with hydroxyl radicals led to some unusual competition plots. The molecules from the arylpropionic family of drugs: ibuprofen, flurbiprofen, ketoprofen and naproxen produced the linear relationship. However, acemetacin, diclofenac Na, flufenamic acid, indometacin, niflumic acid, tolmetin Na and sulindac presented non linear competition plots manifesting at relatively low drug concentrations. This effect was corrected by increasing DR concentrations from 2.8 mM to 15 mM. The modification did not affect rate constants values for those derivatives which already presented a linear plot at 2.8 mM, but allowed to calculate rate constants for other compounds. It is suggested that the experimental conditions have to be adapted particularly for those derivatives with a relatively high rate constant for reaction with the radical species. The oxicam derivatives (tenoxicam and piroxicam) presented another kind of deviation that revealed a prooxidant effect in this system: non linear plots were also observed at relatively low drug concentrations, but in the opposite direction than for the other molecules. This last effect was independent of DR concentration but could be corrected by increasing ascorbate concentration in the system.

KEY WORDS: Deoxyribose assay, hydroxyl radical, antiinflammatory drugs, free radicals, rate constants, antioxidant, prooxidant.

Abbreviations: DR, Deoxyribose; NSAID, non steroidal antiinflammatory drug; MDA, malondialdehyde; TBA, thiobarbituric acid.

INTRODUCTION

Beside inhibiting the synthesis of prostaglandins, non steroidal antiinflammatory drugs (NSAID) were also reported to possess antioxidant properties that rely on different mechanisms.[1–4] However, their activity considerably vary depending on the system used to assess these effects.[1] In order to get a general view of such properties, we re-examined the reactivity of therapeutically used NSAIDs in selected in vitro systems. In this study, the scavenging of hydroxyl radicals (·OH) was considered.[5] Among available techniques, the deoxyribose (DR) method, first introduced in 1981 by Halliwell and

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Gutteridge and further developed in collaboration with Aruoma,[6,7] looked particularly attractive. However, when applying it, we observed several problems that needed further investigation. The present report describes two different cases of non linearity in competition plots that could be solved by modification of experimental conditions.

MATERIALS AND METHODS

Chemicals

2-Deoxy-d-ribose was obtained from Sigma chemicals (Bornem, Belgium). The others reagents used (Merck Belgolabo, Overijse, Belgium) were of high purity. The drugs were in pure form and donated by their respective manufacturers: flurbiprofen and ibuprofen (Boots), ketoprofen and acemetacin (Rhone Poulenc Rorer), naproxen (Sarva Synthex), diclofenac Na (Ciba Geigy), flufenamic acid (Parke Davis), indometacin and sulindac (Merck, Sharp and Dohme), niflumic acid (Upsa Medica), tolmetin Na (Cilag), piroxicam (Pfizer) and tenoxicam (Roche).

Deoxyribose method

The method was first used as described.[6,7] Mixtures contained in a final volume of 1.2 ml: DR (2.8 mM), a pH 7.4 KH_2PO_4/KOH buffer (20 mM), $FeCl_3$ (20 μM), EDTA (100 μM), H_2O_2 (2.8 mM) and ascorbic acid (100 μM). Drugs (0 to 0.8 mM) were dissolved in Na_2CO_3 solutions of the lowest possible concentration and then rapidly adjusted to pH 7.4. $FeCl_3$ and ascorbate solutions were made up in deoxygenated water just before use. $FeCl_3$ and EDTA were premixed prior to addition to the reaction mixture. Ascorbic acid was added to start the reaction. Reaction mixtures were incubated at 37°C for 1 h. After addition of 1 ml of thiobarbituric acid (TBA) 1 % (w/v) in 0.05 mM NaOH and 1 ml of trichloroacetic acid 2.8 % in water, the mixture was heated at 100°C for 20 min. The pink chromogen that progressively developed was then measured at 532 nm after cooling. In the modified procedure allowing determination of rate constants for non linear competition plots obtained with some drugs, DR concentration was raised up to 15 mM, but the incubation time was reduced to 10 min.

The prooxidant activity of drugs was assessed according to Aruoma *et al.* [8–11] The reaction mixtures contained: DR (2.8 mM), a KH_2PO_4/KOH buffer at pH 7.4 (20 mM), H_2O_2 (2.8 mM), $FeCl_3$ (20 μM) premixed with EDTA (100 μM), and 25, 50 or 100 μM of derivative tested dissolved in water, added to start the reaction. Alternatively, ascorbate (50 or 100μM) where used, was added to start the reaction. For the determination of rate constants of oxicams, the modified DR method was used, but ascorbic acid concentration was increased up to 200 μM and 2 ml water were added before absorbance reading.

RESULTS

Deoxyribose assay for antiinflammatory drugs in original conditions

The DR assay is based on the assumption that the initial attack of the radical on DR is the rate-determining step in the formation of a product that leads to malondialdehyde (MDA) the concentration of which is determined by spectrophotometry at 532 nm after reaction with thiobarbituric acid (TBA).[6,7] Starting from Fe(III) ions and ascorbate,

Fe(II) ions are produced which react with H_2O_2 in a Fenton-type reaction to form ·OH radicals. In a reaction mixture containing DR and a scavenging molecule (S) able to compete for ·OH radicals, an expression of 1/A can be derived:

$$1/A = 1/A° + k_S[S]/k_{DR}[DR]A°$$

where k_{DR} and k_S are the respective second order rate constants for DR and the scavenger molecule reactions with ·OH, A is the absorbance obtained at the end of the experiment, taken as a measure of the rate of reaction, and A° is the absorbance measured in the absence of S. Hence, a plot of 1/A against [S] should give a straight line of slope equal to $k_S/k_{DR}[DR]A°$ with an intercept of the y-axis of 1/A°. Considering that the value for k_{DR} is known from pulse radiolysis experiments (3.1×10^9 M^{-1} sec^{-1}), k_s can be calculated from the slope of the experimental line.[6,7]

All antiinflammatory drugs tested were able to inhibit to some extent DR degradation in the original conditions.[6,7] The derivatives of the arylpropionic family, i.e. flurbiprofen, ibuprofen, ketoprofen and naproxen, produced perfectly linear competition graphs suitable to the calculation of rate constants (Table 1). However, unusual cases of competition plots were observed for the remaining drugs. A first kind of problem was observed with other drugs except oxicams. Indeed, although a straight line could be drawn with the absorbance points corresponding to mixtures containing drugs concentrations from 0.1 (sometimes 0.2) to 0.8 mM, the 1/A° point seemed systematically too low. A progressive incurvation of the competition plots was noted at drug concentrations below 0.1 mM, such as indicated (for niflumic acid) in Figure 1. Other drugs presenting the same kind of problem gave quite similar plots, but the intensity of the phenomenon differed: it was quite moderate with diclofenac, more important with acemetacin, niflumic acid and tolmetin Na, and the most pronounced with flufenamic acid, indometacin and sulindac. The second kind of unusual competition plot was observed with the two oxicams: a perfect line could again be calculated with drug concentrations ranging from 0.2 to 0.8 mM, but the point corresponding to

TABLE 1

Hydroxyl radical scavenging activity of non steroidal anti-inflammatory drugs (second order rate constants for reaction with ·OH, Ks) determined by the deoxyribose assay using two different DR concentrations (2.8 and 15 mM). Ascorbate concentration was raised from 100 to 200 μM for oxicams. Values are mean ± SD (n = 2 to 4). The absence of values indicates a non linear competition plot. Literature values are from Aruoma and Halliwell[11] and were obtained with 2.8 mM DR.

	Second order rate constant, 10^{10} M^{-1} s^{-1}		
Drug	Ks, 2.8 mM	Ks, 15 mM	Ks, lit.[11]
Flurbiprofen	0.8 ± 0.1	0.78 ± 0.09	ND
Ibuprofen	1.8 ± 0.2	1.3 ± 0.1	1.5–1.8
Ketoprofen	0.79 ± 0.06	0.7 ± 0.1	0.7–1.0
Naproxen	0.91 ± 0.05	1.119 ± 0.006	1.2–2.2
Acemetacin	–	1.6 ± 0.2	ND
Diclofenac Na	–	1.1 ± 0.2	1.0–2.4
Flufenamic acid	–	2.94 ± 0.07	1.3–3.9
Indometacin	–	1.8 ± 0.1	1.0–1.2
Niflumic acid	–	1.7 ± 0.2	ND
Sulindac	–	2.11 ± 0.08	ND
Tolmetin Na	–	1.0 ± 0.2	ND
Piroxicam	–	1.17 ± 0.04	0.5–0.7
Tenoxicam	–	0.8 ± 0.1	ND

ND = not determined

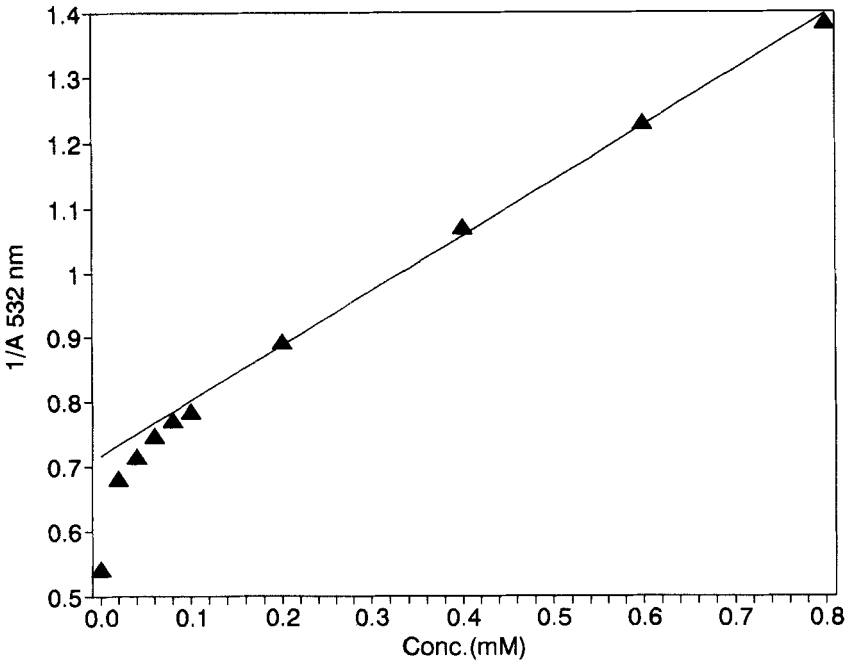

FIGURE 1 Inhibition of deoxyribose degradation by niflumic acid. Experiments were performed as described in the experimental part and with a DR concentration of 2.8 mM.

the absorbance measured in the absence of the drug was this time above the experimental competition plot. Figure 2 (for tenoxicam) shows the progressive incurvation of the plot at low drug concentration occurring in the opposite direction than in Figure 1.

Study of interferences and effects of changing deoxyribose concentration

After an unsuccessful examination of several factors known to interfere with the DR assay,[6,7] the conditions of the competition assay were reconsidered. A significant effect on the shapes of the graphs presenting the first kind of incurvation was finally obtained by increasing DR concentration. Figure 3 reports the effect of initial DR concentration on its degradation by ·OH: a sharp increase in the absorbances as a function of DR concentration was observed at concentrations lower than 5 mM. Then, the increase was more moderate reaching a plateau with relatively stable values between 15 to 30 mM. When adopting the value of 15 mM to perform the assay, the competition graph obtained became linear, such as indicated for niflumic acid in Figure 4. Similar observations were done for other investigated drugs. Absorbances, which increased as a consequence of the modification, had to be maintained within a measurable range by reducing the incubation time from 60 to 10 min. Table 1 reports the values that were obtained for the antiinflammatory molecules except the oxicams for which the competition plots were not linearized.

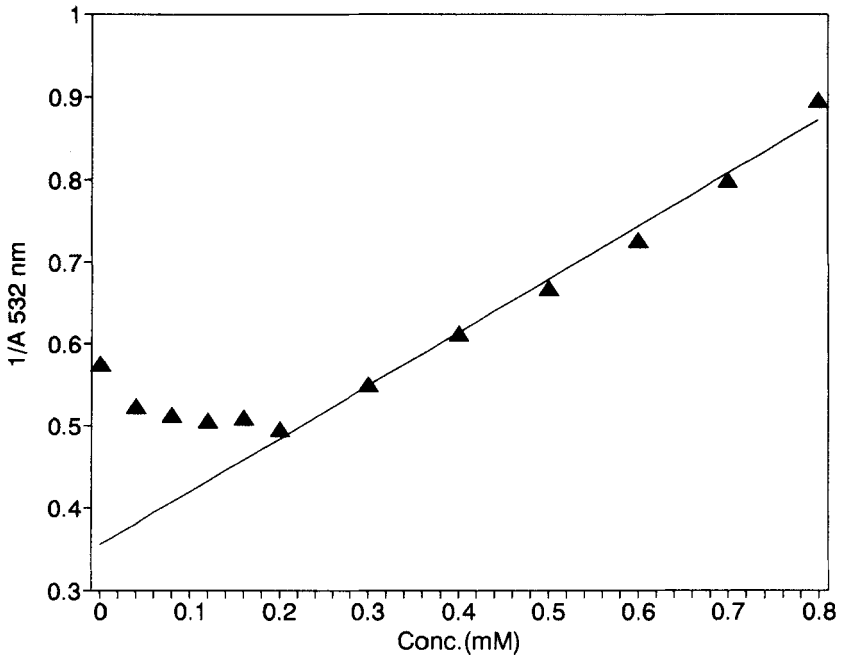

FIGURE 2 Inhibition of deoxyribose degradation by tenoxicam. Experiments were performed as described in the experimental part and with a DR concentration of 2.8 mM.

Pro-oxidant effects of oxicams

The case of the oxicams was suggestive of a prooxidant effect that was documented according to a modification of the DR assay as proposed by Aruoma *et al.*[8–10] Table 2 shows that the two oxicam drugs were able to increase in a dose-dependent manner the damage caused by ·OH on DR such as demonstrated by the increase in absorbance values. This occurred in the absence of ascorbate and suggested that the oxicams could act in some way in the same manner than ascorbate in reducing Fe(III) ions. Another modification in the experimental conditions of the DR test was then examined: increasing ascorbate concentration up to 200 μM led to a complete linearization of the curve allowing to calculate rate constants (Figure 5 and Table 1).

DISCUSSION

Although the DR assay[6,7] seems rather simple to perform, it is not easy to interpret mechanistically as it is based on complex mechanisms which have already been largely discussed.[12–16] As far as we know, non linear competition plots here reported and their resolution by modification of the experimental conditions are original observations.

Adjustment of DR concentration was investigated by Halliwell *et al.*[6] who reported that the rate constants were unaffected by DR concentration if superior or equal to 2.8 mM and consequently adopted this last value for the system. The radicals participating in DR degradation are essentially produced by Fe(II) linked to EDTA and have to migrate a minute distance before they attack DR, the detector molecule.[17] Any

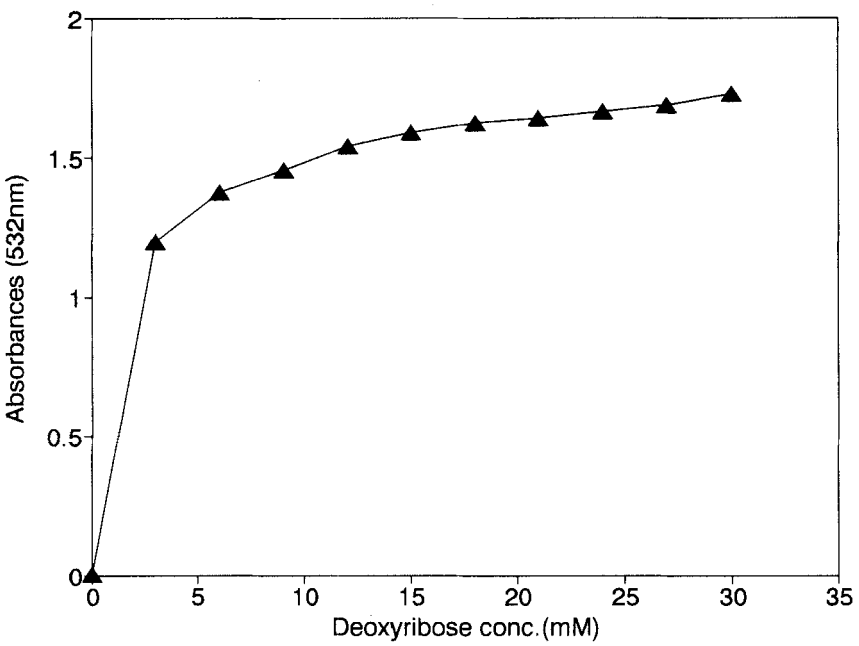

FIGURE 3 Deoxyribose degradation by hydroxyl radicals as a function of DR concentration in the deoxyribose assay.

competition with a scavenging molecule for this species will therefore occur before the radical reaches DR. When examining the extent of DR degradation as a function of DR concentration, we demonstrated first a sharp increase followed by a slowing down of the tendency and ending by a plateau. This means that above a certain DR concentration a maximum rate of degradation is reached and that essentially all the ·OH generated in the reaction are intercepted by the detector molecule. This observation confirmed data obtained in a slightly different system where ·OH are directly generated from Fe(II) salts in the presence of EDTA.[17,18] Therefore, the maximum rate of DR degradation occurred in our conditions at DR concentrations around 15 mM. It is suggested that the part of ·OH that is not taken up by the sugar at 2.8 mM DR directly reacts with the scavenger and decrease the concentration available for the competition with DR, giving rise to non linear competition plots at low drug concentration. The phenomenon attenuates with increasing drug concentrations as radicals that do not participate in the competition progressively become completely scavenged by the drug; the proportionality is then restored between DR damage and drug concentration. The adopted 15 mM DR concentration made it possible to fully intercept ·OH ensuring an efficient competition between the species even at relatively low drug concentrations. Such an hypothesis is corroborated by the observation of differences in the intensity of the deviation among investigated drugs. Results of Table 1 indeed indicate that the derivatives that did not present any problem had a rather low second order rate constant. This suggests that the deviation from linearity observed at 2.8 mM DR is more intense as the drug reacts more rapidly with OH. In other words, drugs reacting more slowly with ·OH give linear competition plots with DR already at 2.8 mM while those reacting more rapidly need higher DR concentrations to give linear graphs.

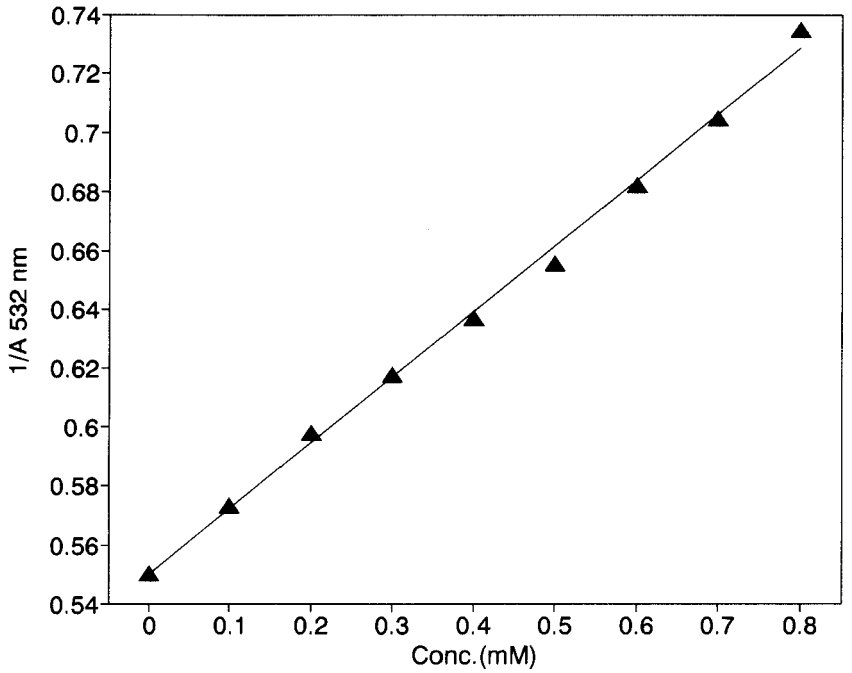

FIGURE 4 Inhibition of deoxyribose degradation by niflumic acid. Experiments were performed as described in the experimental part and with a DR concentration of 15 mM.

TABLE 2
Damage to deoxyribose in the presence of oxicam molecules

Addition to reaction mixture, μM	Damage to deoxyribose, A532 nm	
	Piroxicam	Tenoxicam
none	0.724	0.740
+ ascorbate, 50	1.393	1.381
100	1.868	1.828
+ drug 25	1.321	1.196
50	1.534	1.367
100	1.687	1.549

The first kind of problem could therefore be solved by increasing DR concentration. Not taking into account this phenomenon, for example by drawing a regression line including the 1/A° point, would modify the value of the slope and therefore the rate constant. Another attempt to solve the problem consisting in extrapolating the linear part of the line at 2.8 mM DR till to the y-axis and taking the intercept as 1/A° also leads to inaccurate results as the rate constants calculated in such a way are 17 to 45 % lower than those obtained with the straight lines plots obtained at 15 mM DR (Table 1). There are unfortunately few opportunities to compare the present data with those of other authors. Only Aruoma and Halliwell[11] obtained rate constants that in some cases favourably compare with our results, but they worked at a DR concentration of 2.8 mM (Table 1). A lower level of iron contamination in their reaction mixture could partly explain that they did not observe non linear plots. It is of importance to note

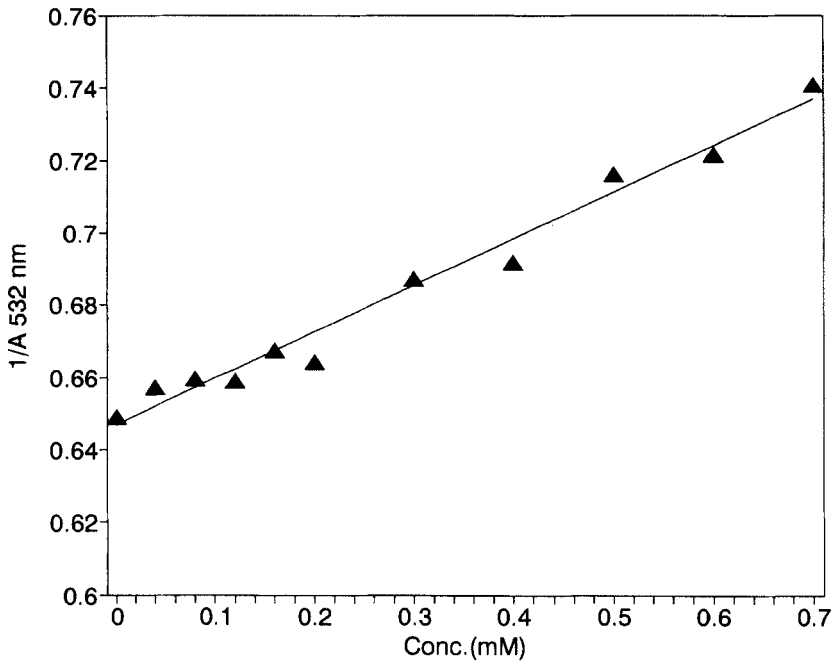

FIGURE 5 Inhibition of deoxyribose degradation by tenoxicam. Experiments were performed as described in the experimental part and with a DR concentration of 15 mM and an ascorbate concentration of 200 μM.

that the rate constants were independent of DR concentration for those drugs for which they were determined at two concentrations and also that the proposed modification did not sensibly modify the precision of the determined rate constants.

The case of oxicams was suggestive of an interference of the drugs with the system causing an increase in oxidative damages to DR which could be detected at low drug concentrations (< 0.2 mM). The occurrence of this phenomenon in the absence of ascorbic acid suggests a prooxidant effect that may be due to the conversion by the drug of Fe(III) to Fe(II), the active form in Fenton reaction.[8] Nevertheless, the damages progressively decreased with increasing drug concentration and the plot became linear thereafter. From that moment, the antioxidant effect of the drug predominates and a linear dose-activity relationship could be obtained. To prevent the effect of oxicams on Fe(III) ions and minimize their competition with the reducing agent, ascorbate concentration in the reaction mixture was raised from 100 to 200 μM. The suppression of the interference indicated that Fe(III) ions are now preferentially reduced by ascorbate and that the drug is again fully available for the competition with DR. Calculated rate constants in modified conditions are reported in Table 1. Similar prooxidant effects were also observed for plant-derived phenolic compounds like gossipol, quercetin and myricetin[8] and for a variety of food additives, e.g. propyl gallate and vanillin.[19]

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