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Evaluation of the free-radical-scavenging activity of diclofenac acid on the free-radical-induced haemolysis of human erythrocytes

You-Zhi Tang and Zai-Qun Liu

Abstract

Free-radical-induced peroxidation in-vivo is regarded as the aetiology of some diseases and free-radical-scavenging drugs, also called antioxidants (AH), have been widely used to overcome oxidative stress. An in-vitro experimental method, 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH)-induced haemolysis of human erythrocytes can be applied to assess the free-radical-scavenging activity of a drug. The major objectives of this work were focused on three aspects. Firstly, introduction of the chemical kinetic deduction of free-radical-initiating reaction to AAPH-induced haemolysis of human erythrocytes, by which the number of free radicals trapped by an antioxidant, n, can be obtained after finding the quantitative relationship between the inhibition period (t_{inh}) and the concentration of the antioxidant, $t_{inh} = (n/R_i)$ [AH]. R_i, the free-radical-initiating rate, was initially confirmed by using α-tocopherol (VE) whose n was taken as 2. Secondly, the free-radical-scavenging activity of diclofenac acid (DaH) and its sodium salt (DaNaH) was assessed. It has been found that DaH and DaNaH protect human erythrocytes against AAPH-induced haemolysis dose-dependently. In particular, the n values of DaH and DaNaH (4.96 and 3.60) were much higher than some traditional antioxidants, such as 6-hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, a water-soluble structural analogue of VE, n = 0.30) and L-ascorbic acid (VC, n = 0.25), and L-ascorbyl-6-laurate (VC-12, a lipophilic structural analogue of VC, n = 1.11). Moreover, the free-radical-scavenging activity of lipophilic antioxidants is higher than the corresponding water-soluble species. Thirdly, the free-radical-scavenging activity of mixed antioxidants, VE + DaH, VC-12 + DaH, Trolox + DaNaH and VC + DaNaH, was revealed. The n value of VC, VC-12, VE and Trolox increase in the case of mixed usage with DaH and DaNaH, implying that diclofenac acid can repair the radical of these antioxidants. Thus, a mutual antioxidant effect between diclofenac acid and these antioxidants prolongs the lifespan of VC, VC-12, VE and Trolox, respectively.

Introduction

Free-radical-scavenging compounds have attracted research interest due to the correlation of free-radical-induced peroxidation in-vivo with many diseases and oxidative stress status (Hegedus 2000; Bao et al 2005; Morello et al 2005; Yu et al 2005), and many evaluation methods have been set up to characterize the free-radical-scavenging activity of the compounds (Antolovich et al 2002; Ivekovic et al 2005; Maldonado et al 2005). We herein apply an in-vitro biological method, 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH)-induced haemolysis (Niki et al 1988; Sato et al 1995), to assess the free-radical-scavenging activity of diclofenac acid (DaH) (Figure 1), along with its water-soluble sodium salt (DaNaH), because the free-radical-scavenging property of the N-H bond has attracted much research attention (Lucarini et al 1999; Gaudiano et al 2003; Tafazoli et al 2005) and the free-radical-scavenging property of diclofenac acid in human erythrocytes is not reported frequently (Parij et al 1998; Gaudiano et al 2003; Amin & Hamza 2005; Paino et al 2005).

The benefits of AAPH-induced haemolysis include the application of the human erythrocytes to reveal the interaction of the free-radical-scavenging drug and in-vivo antioxidant defence system in the erythrocytes membrane. Also, the application of chemical kinetic deduction enables us to calculate the number of trapped free-radicals of a compound, n. The n values of some familiar water-soluble antioxidants (6-hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, a water-soluble structural analogue of α-tocopherol, VE)

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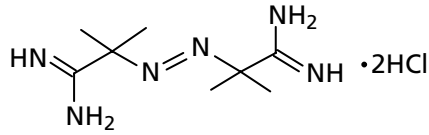
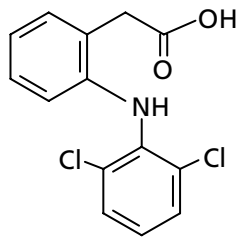
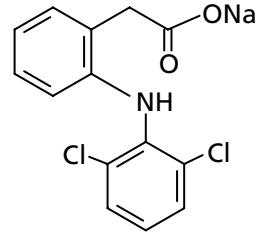


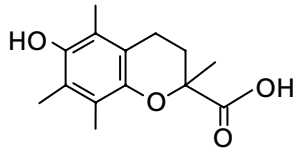
2,2'-azobis(2-amidinopropane hydrochloride), AAPH



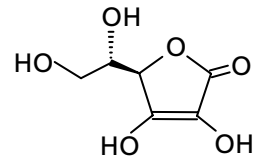
diclofenac acid, DaH



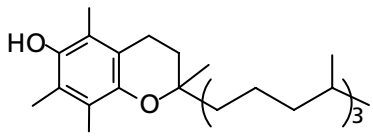
diclofenac sodium salt, DaNaH



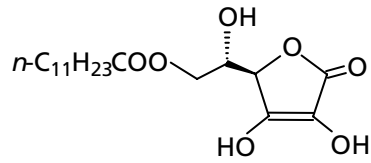
6-hydroxyl-2,5,7,8-tetramethyl
chroman-2-carboxylic acid, Trolox



L-ascorbic acid, VC



α-tocopherol, VE



L-ascorbyl-6-laurate, VC-12

Figure 1 Chemical structures of diclofenac acid, its sodium salt, free-radical-initiator and various antioxidants.

and L-ascorbic acid (VC); Figure 1) and lipophilic antioxidants (VE and L-ascorbyl-6-laurate, VC-12, a lipophilic structural analogue of VC; Figure 1) (Liu et al 1998) were explored in this work and compared with DaNaH and DaH. We also evaluated the mutual free-radical-scavenging activity when water-soluble compounds were mixed (Trolox + DaNaH, VC + DaNaH) or lipophilic ones were mixed (VE + DaH, VC-12 + DaH). This will help us to understand the interactions between these compounds in more detail.

Materials and Methods

Materials

Human erythrocytes collected from healthy subjects were provided by the Red Cross Center for Blood, Changchun, China. After washing three times with phosphate-buffered saline (PBS, 150 mM NaCl, 8.1 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, 50 μM EDTA, pH 7.4 (Zavodnik et al 1999)) to remove plasma, the erythrocytes were centrifuged at 1700 g for exactly 10 min to obtain compact erythrocytes for experimental use (Liu et al 2004). 2,2′-Azobis(2-amidinopropane dihydrochloride) (AAPH) was purchased from Aldrich and dissolved in PBS. VE and Trolox were purchased from Aldrich as well, and VC from Shenyang Chemical Ltd Co. (China). VC-12 and DaH were synthesized following the methods of Cousins et al (1977) and Fu et al (2000), respectively. Because Trolox, VC and DaNaH are water-soluble species, they were dissolved in PBS directly. Since VE, VC-12 and DaH cannot be dissolved in PBS, they were dissolved in dimethyl sulfoxide (DMSO) as the stock solution. It was worth pointing out that the same amount of DMSO

(< 1.0% of the total volume of haemolysis mixture) was contained in all the experiments to avoid its influence on the haemolysis in the case of lipophilic antioxidants used in the experiment (Liu et al 2004).

Expression of haemolysis process

The haemolysis experiment followed the description given in the literature (Niki et al 1988; Sato et al 1995; Zou et al 2001; Liu et al 2003a). Antioxidant stock solution and AAPH solution (30 mM as the final concentration) were added successively to the 3.0% erythrocyte suspensions in PBS (v/v). Then, the above mixture was put into a 37°C thermostatic bath to initiate the haemolysis. Samples were taken from the above mixture at appropriate intervals and centrifuged at 1700 *g* for 5 min to obtain the supernatant. Then the absorbance of the supernatant was determined at 540 nm (May et al 1998). The haemolysis process can be illustrated by the relationship between the absorbance at 540 nm (A) and the incubation time (t) as Figure 2 shows, in which every experiment was repeated three times, and the points in Figures 2 and 3 were the average value from three independent measures within 10% experimental error.

The haemolysis curves in Figures 2 and 3 can be expressed by the Boltzmann equation (Liu et al, 2003b):

$$A = (A_{initial} - A_{final})/(1 + e^{(t-t_0)/dt}) + A_{final} \quad (1)$$

where $A_{initial}$ and A_{final} refer to the absorbance at the beginning and end of the haemolysis, t_0 represents the time for erythrocytes to reach 50% haemolysis and dt indicates the variance of time. We designated t_0 as the lag time of the haemolysis (referred to as t_{lag}) because it not only contains the period during which haemolysis does not take place, but also involves the influence of the antioxidant on the haemolysis rate.

The evaluation of the free-radical-scavenging activity of an antioxidant on the basis of chemical kinetic deduction

The haemolysis ascribes to the attack from the initiating radical ($ROO^{\cdot}$,R=$C(CH_3)_2C(NH_2)$=NH) generated from the decomposition of AAPH (R-N = N-R):

$$R\text{–}N = N\text{–}R \rightarrow 2\varepsilon R^{\cdot} + N_2 + (1\text{–}\varepsilon)R\text{–}R \quad (2)$$

$$R^{\cdot} + O_2 \rightarrow ROO^{\cdot} \quad (3)$$

where ε is the phase-transfer efficiency that reveals the efficiency of $ROO^{\cdot}$ to transfer into the membrane (Bowry & Stocker 1993). Then $ROO^{\cdot}$ attacks the polyunsaturated fatty acid (LH) in membrane (equation 4) to initiate the radical propagation (equations 5 and 6), in which R_i refers to the initiating rate, thus, the generation

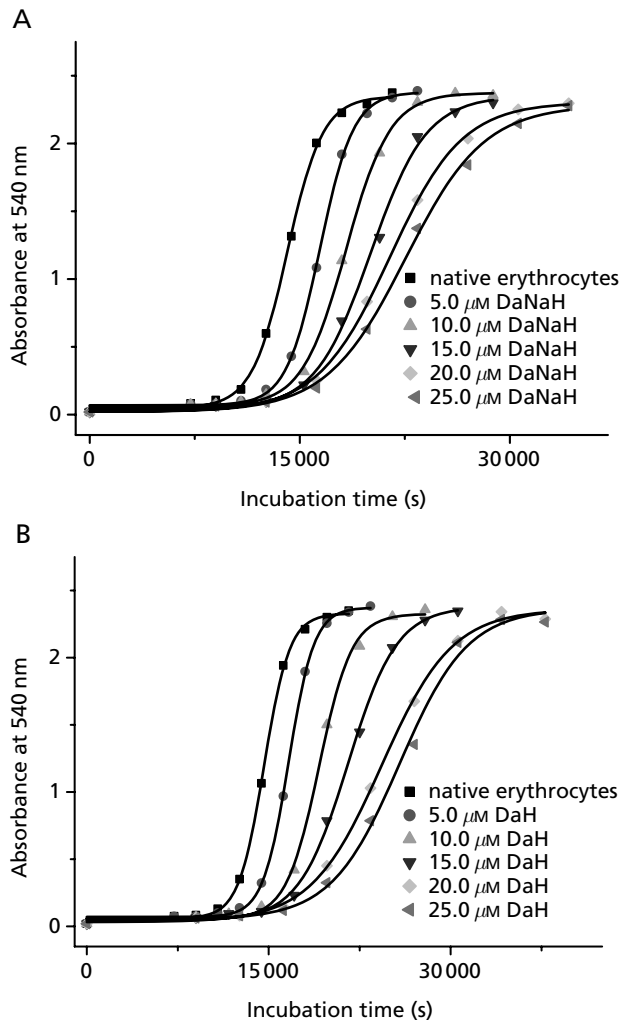


Figure 2 Haemolysis curves of human erythrocytes (3.0% suspension in PBS, pH 7.4) initiated by AAPH (30 mM) at 37°C in the presence of DaNaH (A) and DaH (B) (the haemolysis curves in the presence of Trolox, VE, VC and VC-12 are not shown) at various concentrations. The data points represent the average value from three independent measurements with the experimental error within 10%, and the error bars are not shown for clarity.

of peroxyl radical ($LOO^{\cdot}$) eventually causes oxidizable haemolysis (Ma et al 2000).

$$ROO^{\cdot} + LH \xrightarrow{R_i} ROOH + L^{\cdot} \quad (4)$$

$$L^{\cdot} + O_2 \xrightarrow{fast} LOO^{\cdot} \quad (5)$$

$$LOO^{\cdot} + LH \xrightarrow{k_p} LOOH + L^{\cdot} \quad (6)$$

If an antioxidant (AH) is added to the haemolysis mixture, it interacts with $LOO^{\cdot}$ to form an antioxidant radical ($A^{\cdot}$) (equation 7) that can couple rapidly with another $LOO^{\cdot}$ to form a non-radical product (LOOA) (equation 8); the haemolysis would be inhibited efficiently.

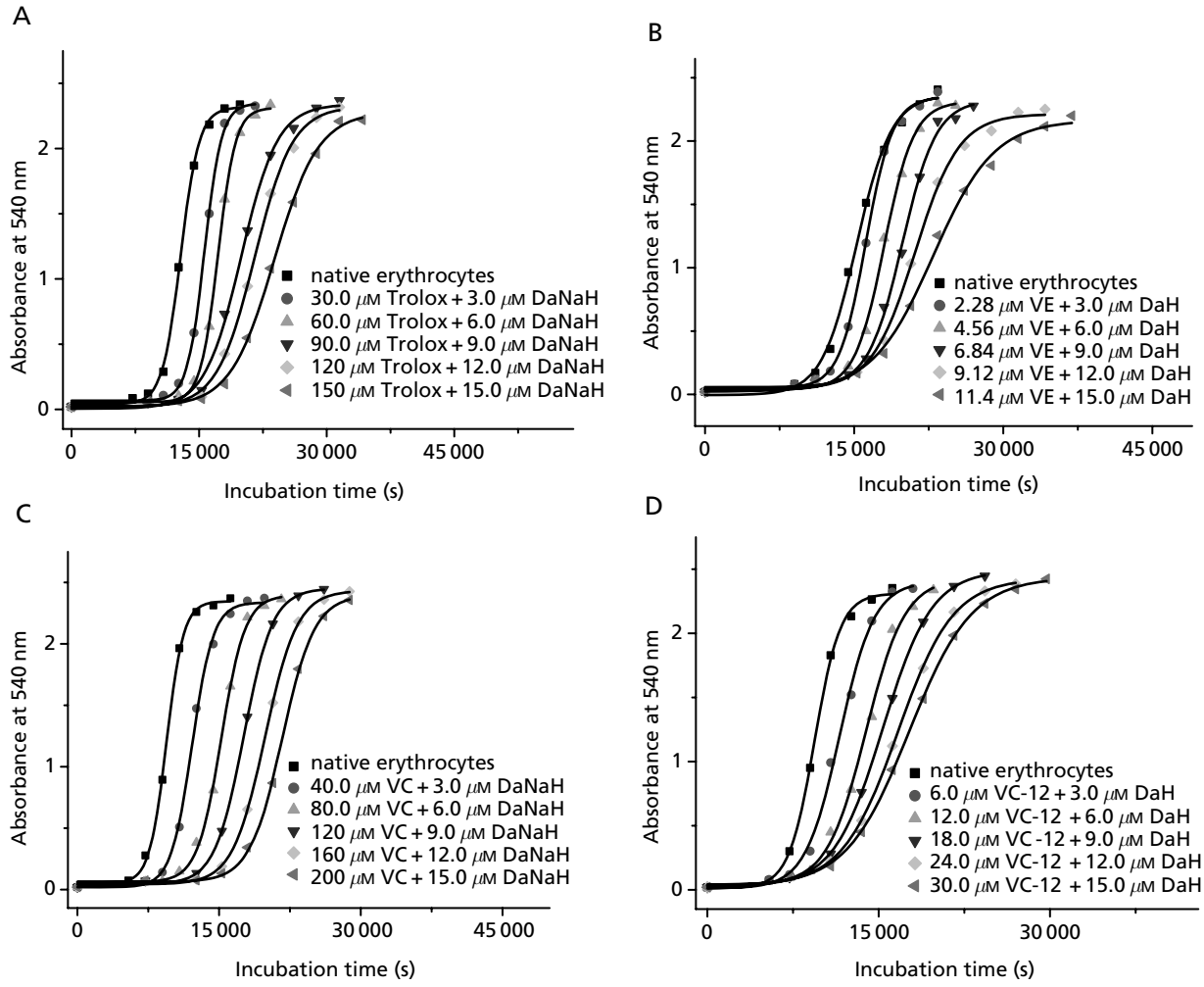


Figure 3 Haemolysis curves of human erythrocytes (3.0% suspension in PBS, pH 7.4) initiated by AAPH (30 mM) at 37 °C in the presence of Trolox + DaNaH (A), VE + DaH (B), VC + DaNaH (C) and VC-12 + DaH (D) at various concentrations. The data points represent the average value from three independent measurements with the experimental error within 10%, and the error bars are not shown for clarity.

$$LOO^{\cdot} + AH \overset{k_{inh}}{\rightarrow} LOOH + A^{\cdot} \quad (7)$$

$$LOO^{\cdot} + A^{\cdot} \overset{fast}{\rightarrow} LOOA \quad (8)$$

To sum up equations 2—8 and treat by the steady-state kinetic deduction to yield the R_i (Fang et al 2002)

$$R_i = (n[AH])/t_{inh} \quad (9)$$

where n is the stoichiometric factor, whose physical meaning can be regarded as the number of $LOO^{\cdot}$ trapped by each AH molecule, and n can be a decimal value (Lucarini et al 1999). t_{inh} refers to the inhibition period generated by the addition of AH. Equation 9 can be expressed equivalently as the $t_{inh} \sim$ [AH] equation:

$$t_{inh} = (n/R_i)[AH] \quad (10)$$

in which the slope, n/R_i, will implicate n with the R_i known. Nevertheless, it is difficult to determine R_i directly. VE is generally selected to be the standard antioxidant whose n value is always taken as 2 (Bowry & Stock 1993). Consequently, R_i can be calculated via the relationship of $t_{inh} \sim$ [VE]. Finally, by the known R_i, the n of other antioxidants can be obtained via the corresponding $t_{inh} \sim$ [AH], hence, the obtained n is a relative value compared with 2 of VE.

Statistical analysis

All the quantitative relationships involving Boltzmann equations and $t_{inh} \sim$ [AH] equations (the correlation coefficients are listed in Table 1 and 2) were performed

Table 1 The quantitative relationships between inhibition periods and concentrations of various antioxidants

Antioxidant	$t_{inh} = (n/R_i)$ [concn (μM)] + B[a]	n	Correlation coefficient
Trolox	$t_{inh} = (28.5 \pm 1.88)$[Trolox] − 427.2	0.30 ± 0.02	0.9914
VE	$t_{inh} = (187.1 \pm 9.12)$ [VE] + 148.7	2.00[b]	0.9958
VC	$t_{inh} = (23.8 \pm 1.06)$ [VC] + 258.3	0.25 ± 0.01	0.9960
VC-12	$t_{inh} = (104.0 \pm 6.96)$ [VC-12] − 35.4	1.11 ± 0.07	0.9912
DaNaH	$t_{inh} = (337.0 \pm 21.33)$ [DaNaH] + 500.4	3.60 ± 0.23	0.9921
DaH	$t_{inh} = (463.9 \pm 15.46)$ [DaH] − 77.0	4.96 ± 0.17	0.9978

[a]The slope in the equation is n/R_i, in which n refers to the number of the radicals trapped by the antioxidant, and R_i is the radical initiating rate. [b]VE is the reference antioxidant and its n value is 2. Thus, $R_i = 1.069 \times 10^{-8}$ M s^{-1}. The n value of other antioxidants can be calculated by the slope × R_i.

Table 2 The influence of the concentrations of two antioxidants on the inhibition periods

Antioxidant	$t_{inh} = (n_{total}/R_i)$ [C_{other} + $C_{diclofenac}$] + B[a]	n_{total}	Correlation coefficient
Trolox + DaNaH	$t_{inh} = (65.4 \pm 2.35)$ [Trolox + DaNaH] + 275.6	0.70 ± 0.03	0.9974
VE + DaH	$t_{inh} = (298.9 \pm 11.56)$ [VE + DaH] − 288.7	3.20 ± 0.12	0.9970
VC + DaNaH	$t_{inh} = (57.6 \pm 2.48)$ [VC + DaNaH] + 363.0	0.62 ± 0.03	0.9963
VC-12+ DaH	$t_{inh} = (184.2 \pm 14.72)$ [VC-12 + DaH] + 555.9	1.97 ± 0.16	0.9875

[a]$R_i = 1.069 \times 10^{-8}$ M s^{-1}.

statistically by one-way analysis of variance using Origin 6.0 professional Software. $P < 0.001$ indicated a significant difference.

Results

Figure 2A, B outlines the haemolysis curves in the presence of DaNaH and DaH, respectively, and t_{lag} in the presence of various concentrations are obtained from equation 1.

The reason why the haemolysis is still lagged even without antioxidant added is due to the endogenous antioxidants in the erythrocyte membrane playing a protective role in the initial period of AAPH-induced haemolysis (Sato et al 1995). To eliminate the influence of the endogenous antioxidants in the erythrocyte membranes from different subjects, the inhibition period, $t_{inh} = t_{lag} - t_{lag0}$, is designated to reflect the function of additive antioxidants, where t_{lag} refers to the lag time of haemolysis in the presence of exogenous antioxidant, while t_{lag0} indicates the lag time derived from the endogenous ones. The quantitative relationship between t_{inh} and the concentration of various antioxidants are listed in Table 1.

To explore the mutual antioxidative activity, water-soluble antioxidant mixtures (Trolox + DaNaH, VC + DaNaH) and lipophilic antioxidant mixtures (VE + DaH, VC-12 + DaH) were applied to protect erythrocytes against AAPH-induced haemolysis, respectively; the haemolysis curves are shown in Figure 3, and the quantitative relationships between t_{inh} and the concentration of Trolox + DaNaH, VC + DaNaH, VE + DaH and VC-12 + DaH are listed in Table 2.

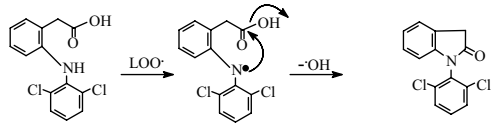
Discussion

The number of free-radicals trapped by the antioxidants

It has been proved that lipid peroxidation in model biomembranes follows the same classical law as that in homogenous solutions (Barclay & Ingold 1980; Barclay 1993). The AAPH-induced oxidizable haemolysis of human erythrocytes is a process of free-radical-induced peroxidation in a real biomembrane. Therefore, chemical kinetic deduction, as discussed as in the Method, will benefit the exploration of the antioxidant activity in detail. The value of > 0.99 for the correlation coefficients in Table 1 demonstrates that all these compounds are dose-dependent antioxidants. Although erythrocytes are suspended in PBS, the oxidizable haemolysis takes place within the membrane that is a lipophilic microenvironment (Ma et al 2000). So, VE, a lipophilic standard antioxidant, is suitable for the reference antioxidant whose n is taken as 2, meaning that one VE molecule can trap two $LOO^{\cdot}$. The R_i can be obtained by $2/(187.1\ s\ \mu M^{-1}) = 1.069 \times 10^{-8}$ M s^{-1}, with the result that

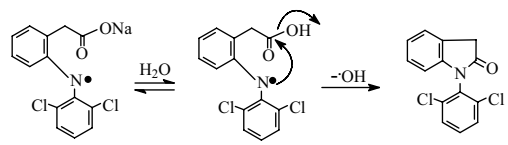
the n of other antioxidants can be calculated by the corresponding slope $\times$ R_i (see Table 1).

It was found that the n values of diclofenac acid, either the water-soluble species (DaNaH) or lipophilic species (DaH), were larger than those of any other antioxidants, demonstrating that the free-radical-scavenging activity of diclofenac acid is much higher than some traditional free-radical-scavengers (i.e., VC and VE). Besides, the n of DaH, 4.96, was larger than that of DaNaH, 3.60, revealing that the free-radical-scavenging activity of the lipophilic species of diclofenac acid was better than its water-soluble species. Although DaH is a carboxylic acid with a pK_a even lower than 4, it is difficult to ionize to form Da^- because it cannot be dissolved in PBS. On the contrary, its lipophilicity may allow it to dissolve in membrane easily, resulting in its free-radical-scavenging efficiency being higher than that of its sodium salt. This phenomenon was also observed with the n values of VE (2.00) and VC-12 (1.11), which were larger than their corresponding water-soluble species, Trolox (0.30) and VC (0.25). It can be regarded that the liposolubility makes lipophilic species penetrate into membranes to protect erythrocytes efficiently, while the water-soluble species just locate in PBS. The protective function of water-soluble species just takes place at the surface of membranes though their structure is similar. Furthermore, to understand the above result, 1-(2,6-dichlorophenyl)indolin-2-one, an oxidative product derived from $OH^{\cdot}$ (Gaudiano et al 2003), was introduced in our discussion because either $LOO^{\cdot}$ or $OH^{\cdot}$ is an oxidized radical. Thus, the free-radical-scavenging procedure of diclofenac acid can be described by equation 11:



1-(2,6-dichlorophenyl)indolin-2-one (11)

If DaNaH is available for scavenging free-radicals, the DaNaH radical cannot convert to the final product because $-ONa$ cannot be eliminated directly. The DaNaH radical should convert to the DaH radical for the sake of elimination of the hydroxyl radical ($^{\cdot}OH$) as equation 12 shows.

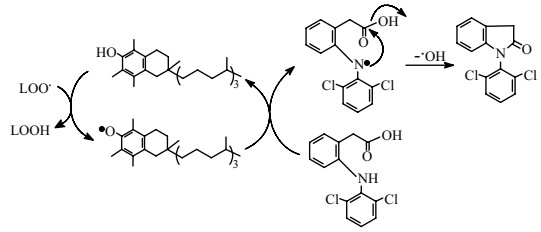


(12)

The equilibrium between DaNaH and DaH may slow down the reaction of eliminating $^{\cdot}OH$. So, the above deduction will benefit the understanding of the result that the free-radical-scavenging activity of DaNaH is lower than that of DaH.

The number of free-radicals trapped by mixed antioxidants

To reveal the effect of solubility of antioxidants on the free-radical-scavenging activity, the relationship between t_{inh} and the sum of the concentration of other antioxidants and diclofenac acid ($C_{other} + C_{diclofenac}$) are listed in Table 2. The value of >0.98 of the correlation coefficients in Table 2 indicates that t_{inh} is proportional to ($C_{other} + C_{diclofenac}$). The R_i is still taken as 1.069×10^{-8} M s^{-1}, and the number of the mixed antioxidants, n_{total}, can be calculated by the corresponding slope $\times$ R_i and are listed in Table 2 as well. It was found that n_{total} values of lipophilic mixtures (VE + DaH (3.20), VC-12 + DaH (1.97)) were larger than those of their corresponding water-soluble mixtures (Trolox + DaNaH (0.70), VC + DaNaH (0.62)). This fact also supports our implication that lipophilic antioxidants permeate into membranes easily and exhibit higher free-radical-scavenging activity in this experimental system. Furthermore, with the help of DaH and DaNaH, the n values of VC, VC-12, VE and Trolox increase remarkably. For example, the n value of VE, 2.00, increases to 3.20 in the presence of DaH, and the n value of VC, 0.25, increases to 0.62 in the presence of DaNaH. This implies that diclofenac acid can recycle the radicals of other antioxidants. Taking VE + DaH as an example, the VE radical derived from the oxidation of $LOO^{\cdot}$ can be repaired by DaH as equation 13 shows, and DaH radical converts to 1-(2,6-dichlorophenyl)indolin-2-one finally.



(13)

The reason why the n value of DaNaH and DaH used in combination with other antioxidants is far less than that for them used alone is due to diclofenac acid repairing the radical of other antioxidants rather than trapping $LOO^{\cdot}$ directly.

Conclusion

To sum up, these findings show that AAPH-induced haemolysis of human erythrocytes acts as an in-vitro experimental model that can be applied conveniently to evaluate the free-radical-scavenging activity of drugs. Diclofenac acid, either its lipophilic species or water-soluble species, can be dose-dependent free-radical-scavenging drugs with high activity. Especially, they can also mutually protect erythrocytes with Trolox, VE, VC and VC-12. Moreover, the free-radical-scavenging activity of DaH is higher than that of DaNaH. This information may be useful in the pharmaceutical usage of diclofenac acids.

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