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Protective effects of curcumin and its analogues against free radical-induced oxidative haemolysis of human red blood cells

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Abstract

Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, **1**), is a yellow ingredient isolated from turmeric (*curcumin longa*). It has been shown to exhibit a variety of biological activities, including antioxidative activity. In order to find more active antioxidants with **1** as the lead compound, we synthesized curcumin analogues, namely 1-(4-hydroxy-3- methoxyphenyl)-7-(4-hydroxyphenyl)-1,6-heptadiene-3,5-dione (**2**), 1-(3,4-dimethoxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (**3**), 1,7-bis(3,4-dimethoxyphenyl)-1,6-heptadiene-3,5-dione (**4**), 1-(3,4-dihydroxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (**5**), 1,7-bis(3,4-dihydroxyphenyl)-1,6-heptadiene-3,5-dione (**6**), and 1,7-bis-(4-hydroxyphenyl)-1,6-heptadiene-3,5-dione (**7**), and evaluated their antioxidative activity. The in vitro oxidative haemolysis of human red blood cells (RBCs) was used as a model to study the free radical-induced damage of biological membranes and the protective effects of these curcumin analogues. It was found that these compounds, except **4**, could effectively inhibit the free radical induced oxidative haemolysis of RBCs by H-atom abstraction from the phenolic groups. Compounds **5** and **6** which bear *ortho*-diphenoxyl functionality exhibited markedly higher anti-haemolysis activity than curcumin and other analogues, as well as than α-tocopherol (vitamin E).

Keywords: Lipid peroxidation; Antioxidation; Haemolysis; Curcumin; Phenolic antioxidants; Structure/activity relationship

1. Introduction

Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, **1**) is a naturally occurring phenolic compound isolated as a yellow pigment from turmeric (dry rhizomes of *curcumin longa*) which is commonly used as a spice and food colorant (Buescher & Yang, 2000). This compound has been reported to possess a variety of biological and pharmacological activities, including antioxidative (Daniel, Limson, Dairam, Watkins, & Daya, 2004; Naidu & Thippeswamy, 2002; Priyadarsini et al., 2003; Rukkumani, Balasubashini, & Menon, 2003; Venkatesan & Rao, 2000), anti-inflammatory (Chainani-Wu, 2003), anticarcinogenic (Aqqarwal, Kumar, & Bharti, 2003; Cheng et al., 2001; Lin & Lin-Shia, 2001; Shukla, Aroro, & Taneja, 2002), and anti-HIV (Mazumder, Raghavan, Weinstein, Kohn, & Pommier, 1995). The desirable cancer preventive or putative therapeutic properties of curcumin have also been considered to be associated with its antioxidant properties (Huang et al., 1991; Kelly, Xu, Alexander, & Loo, 2001; Ruby, Kuttan, Dinesh, Rajasekharan, & Kuttan, 1995; Skrzypczak-Jankun, McCabe, Selman, & Jankun, 2000), since free radical mediated peroxidation of membrane lipids and oxidative damage of DNA are believed to be associated with a variety of chronic health problems, such as cancer, atherosclerosis, neurodegenerative diseases and aging (Barnham, Masters, & Bush, 2004; Cooke, Evans,

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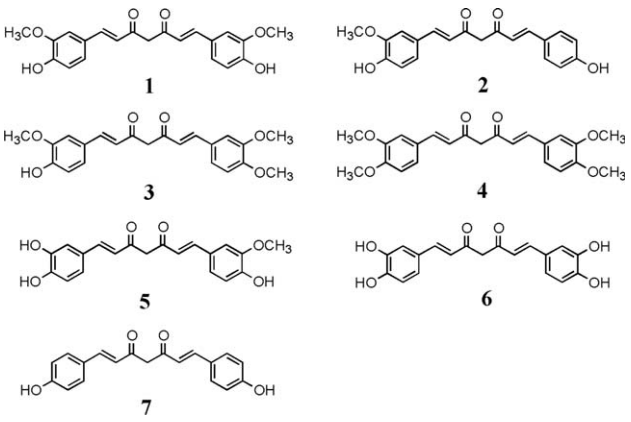
Dizdaroglu, & Lunez, 2003; Finkel & Holbrook, 2000; Halliwell & Gutteridge, 1999; Perwez Hussain, Hofseth, & Harris, 2003; Marnett, 2000). Therefore, the past few years have witnessed intense research devoted to the antioxidant activity of curcumin (Daniel et al., 2004; Huang et al., 1991; Kelly et al., 2001; Naidu & Thippeswamy, 2002; Priyadarsini et al., 2003; Ruby et al., 1995; Rukkumani et al., 2003; Skrzypczak-Jankun et al., 2000; Venkatesan & Rao, 2000; Wright, 2002). On our ongoing research project on kinetics and mechanisms of natural antioxidants (Cai et al., 2002; Chen, Zhou, Yang, Wu, & Liu, 2001; Hou, Zhou, Yang, & Liu, 2004a, Hou, Zhou, Yang, & Liu, 2004b; Liu, Ma, Zhou, Yang, & Liu, 2000; Zhou et al., 2000; Zhou, Yang, & Liu, 2004; Zhou, Miao, Yang, & Liu, 2005a, Zhou, Wu, Yang, & Liu, 2005b), we recently found that simple structural modification of resveratrol, which is an antioxidative component in red wine, could significantly enhance its antioxidative activity (Cai, Fang, Ma, Yang, & Liu, 2003; Fang et al., 2002) and cytotoxicity against cancer cells (Cai et al., 2004). This motivated us to use curcumin as a lead compound to design more active potential antioxidants and chemopreventive agents against cancer. Reported herein is the synthesis of a set of curcumin analogues and an in vitro study of their protective effects on free radical-induced haemolysis of human red blood cells (RBCs). RBC membranes are rich in polyunsaturated fatty acids which are very susceptible to free radical-mediated peroxidation, leading to damage of the membrane and haemolysis. The peroxidation was initiated by 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH) that could decompose at physiological temperature and generate alkyl radicals to initiate lipid peroxidation (Eq. (1), vide infra). Since AAPH is water-soluble and the generation rate of free radicals from the decomposition of AAPH can be easily controlled and measured, it has been extensively used as a free radical initiator for biological and related studies (Cai et al., 2002; Cai et al., 2003; Fang et al., 2002; Hou et al., 2004a, 2004b; Liu et al., 2000; Tedesco et al., 2000; Zhou et al., 2000; Zhou et al., 2004; Zhou et al., 2005a, 2005b) and the AAPH-induced haemolysis provides a good experimental approach for studying free radical-induced membrane damage (Ma, Liu, Zhou, Yang, & Liu, 2000; Niki et al., 1988). The compounds studied were curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, **1**), 1-(4-hydroxy-3-methoxyphenyl)-7-(4-hydroxyphenyl)-1,6-heptadiene-3,5-dione (**2**), 1-(3,4-dimethoxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (**3**), 1,7-bis(3,4-dimethoxyphenyl)-1,6-heptadiene-3,5-dione (**4**), 1-(3,4-dihydroxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (**5**), 1,7-bis(3,4-dihydroxyphenyl)-1,6-heptadiene-3,5-dione **(6)**, and 1,7-bis-(4-hydroxyphenyl)-1,6-heptadiene-3,5-dione **(7)**.



2. Materials and methods

2.1. Materials

Curcumin and its analogues **(1–7)** were synthesized by condensation of 2,4-pentanedione with two equivalents of substituted benzaldehyde based on the available methods (Mazumder et al., 1997; Roughley & Whiting, 1973). Generally, 2,4-pentanedione (1.0 g, 0.01 mol) and boron oxide (0.49 g, 0.007 mol) were dissolved in EtOAc (10 ml) and stirred for 0.5 h at 40 °C followed by addition of the corresponding benzaldehyde (0.02 mol) and tributyl borate (4.6 g, 0.02 mol) and stirred for an additional 0.5 h. Then *n*-butylamine (1 ml) in EtOAc (10 ml) was added dropwise during 30 min. After further stirring for 4 h at 40 °C the mixture was allowed to stand overnight to complete the reaction. The mixture was hydrolyzed by HCl (0.4 N, 15 ml) and the aqueous layer extracted three times with EtOAc. The combined organic layers were washed with water and dried over Na_2SO_4. After removal of the solvent under reduced pressure, the residual paste was purified by column chromatography (silica gel, cyclohexane–EtOAc) and recrystallized from EtOH to give pure **1–4** and **7**. Compounds **5** and **6** were obtained by demethylation of **1** with $AlCl_3$-pyridine, as described in the literature (Mazumder et al., 1997).

1,7-Bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (curcumin, **1**): 48% yield from vanillin (3.0 g, 0.02 mol), m.p. 184–185 °C [lit. (Mazumder et al., 1997), m.p. 182–183 °C]. ^{1}H NMR [400 MHz, $(CD_3)_2CO$]: δ 3.91 (s, 6H), 5.97 (s, 1H), 6.68 (d, $J = 15.8$ Hz, 2H), 6.88 (d, $J = 8.2$ Hz, 2H),7.17 (d, $J = 8.2$ Hz, 2H), 7.32 (s, 2H), 7.59 (d, $J = 15.8$ Hz, 2H). EIMS: m/z (%) 368 ($M^{+\cdot}$, 45).

1-(4-hydroxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (**2**): 16% yield from vanillin (1.5 g, 0.01 mol) and 4-hydroxybenzaldehyde (1.2 g, 0.01 mol), m.p. 170–172 °C [lit. (Mazumder et al.,

1997), m.p. 170–171 °C]. ^{1}H NMR [400 MHz, $CDCl_3$]: δ 3.84 (s, 3H), 5.93 (s, 1H), 6.60 (d, $J = 15.6$ Hz, 1H), 6.66 (d, $J = 16.4$ Hz, 1H), 6.82 (d, $J = 8.0$ Hz, 1H), 6.83 (d, $J = 8.0$ Hz, 2H), 7.10 (dd, $J = 1.6$, 8.0 Hz, 1H), 7.26 (d, $J = 1.6$ Hz, 1H), 7.49 (d, $J = 8.0$ Hz, 1H), 7.52 (d, $J = 16.4$ Hz, 1H), 7.53 (d, $J = 15.8$ Hz, 1H). EIMS (m/z, %): 338 ($M^{+\cdot}$, 1).

1-(4-hydroxy-3-methoxyphenyl)-7-(3,4-dimethoxyphenyl)-1,6-heptadiene-3,5-dione (**3**): 14% yield from vanillin (1.5 g, 0.01 mol) and 3,4-dimethoxybenzaldehyde (1.7 g, 0.01 mol), m.p. 130–132 [lit. (Jovanovic, Boone, Steenken, Trinoga, & Kaskey, 2001) dark yellow viscous oil]. ^{1}H NMR [400 MHz, $(CD_3)_2CO$]: δ 3.85 (s, 3H), 3.87 (s, 3H), 3.90 (s, 3H), 5.61 (s, 1H), 6.71 (d, $J = 16.4$ Hz, 1H), 6.75 (d, $J = 16.4$ Hz, 1H), 6.87 (d, $J = 8.4$ Hz, 1H), 6.70 (d, $J = 8.0$ Hz, 1H), 7.16 (d, $J = 8.4$ Hz, 1H), 7.23 (d, $J = 8.4$ Hz, 1H), 7.31 (s, 1H), 7.32 (s, 1H), 7.58 (d, $J = 16.0$ Hz, 2H). EIMS (m/z, %): 382 ($M^{+\cdot}$, 2).

1,7-Bis(3,4-dimethoxyphenyl)-1,6-heptadiene-3,5-dione (**4**): 52% yield from 3,4-methoxy-benzaldehyde (3.3 g, 0.02 mol), m.p. 130–131°C [lit. (Roughley & Whiting, 1973), m.p. 128–130°C]. ^{1}H NMR [400 MHz, $CDCl_3$]:δ 3.92, (s, 6H), 3.94 (s, 6H), 5.82 (s, 1H), 6.50 (d, $J = 16.0$ Hz, 2H), 6.88 (d, $J = 8.0$ Hz, 2H), 7.08 (d, $J = 2.0$ Hz, 2H), 7.15 (dd, $J = 2.0$, 8.0 Hz, 2H), 7.61 (d, $J = 16.0$ Hz, 2H). EIMS (m/z, %): 396 ($M^{+\cdot}$, 31).

1-(3,4-dihydroxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (**5**): 40% yield from **1** (5.5 g, 0,015 mol), m.p. 166–168^{o}C [lit. (Mazumder et al., 1997), m.p. 165–167 °C]. ^{1}H NMR [300 MHz, $(CD_3)_2CO$]: δ 3.92 (s, 3H), 5.97 (s, 1H), 6.58 (d, $J =$ 15.9 Hz, 1H), 6.69 (d, $J = 15.9$ Hz, 1H), 6.86 (d, $J = 8$ Hz, 2H), 7.02 (d, $J = 8.1$ Hz, 1H), 7.15 (d, $J = 8.1$ Hz, 1H), 7.16 (s, 1H), 7.31 (s, 1H), 7.51 (d, $J = 16.5$ Hz, 1H), 7.62 (d, $J = 16.8$ Hz, 1H). EIMS (m/z, %): 354 ($M^{+\cdot}$, 2).

1,7-Bis(3,4-dihydroxyphenyl)-1,6-heptadiene-3,5-dione (**6**): 25% yield from **1** (5.5 g, 0,015 mol), m.p. 304–306 °C decomp. [lit. (Mazumder et al., 1997), m.p. 306–308 °C decomp.]. ^{1}H NMR [200 MHz, $(CD_3)_2CO$]: δ 5.99 (s, 1H), 6.50 (d, $J = 15.8$ Hz, 2H), 6.88 (d, $J = 8.6$ Hz, 2H), 7.07 (d, $J = 8.6$ Hz, 2H), 7.19 (s, 2H), 7.53 (d, $J = 15.8$ Hz, 2H). FAB/MS (m/z): 341 $(M+1)^{+\cdot}$.

1,7-Bis(4-hydroxyphenyl)-1,6-heptadiene-3,5-dione (**7**): 53% yield from 4-hydroxy-benzaldehyde (2.4 g, 0.02 mol), m.p. 221–222 °C [lit. (Mazumder et al., 1997), m.p. 224 °C]. ^{1}H NMR [400 MHz, $(CD_3)_2CO$]: δ 5.99 (s, 1H), 6.66 (d, $J = 16.0$ Hz, 2H), 6.89 (dd, $J = 8.0$, 2.2 Hz, 4H), 7.56 (d, $J = 8.0$ Hz, 4H), 7.59 (d, $J = 16.0$ Hz, 2H). EIMS (m/z, %): 308. ($M^{+\cdot}$, 28).

2,2′-Azobis(2-amidinopropane hydrochloride) (AAPH) and DL-α-tocopherol (Biochemical reagent, >99.9%) were purchased from Aldrich and Sigma, respectively and used as received. Other chemicals used were of analytical grade.

2.2. Preparation of RBC

Human red blood cells were separated from heparinized blood that was drawn from a healthy donor. The blood was centrifuged at 2000 rpm for 10 min to separate the RBCs from plasma, then the RBCs were washed three times with phosphate-buffered saline (PBS) at pH 7.4. During the last washing the cells were centrifuged at exactly 2000 rpm for 10 min to obtain a constantly packed cell volume.

2.3. Assay for haemolysis

The 5% suspension of RBCs in PBS (pH 7.4) was incubated under air atmosphere at 37 °C for 5 min; into this a PBS solution of AAPH was added to initiate haemolysis. The reaction mixture was shaken gently while being incubated at 37 °C. The extent of haemolysis was determined spectrophotometrically as described previously (Kuang, Wang, Zheng, Liu, & Liu, 1994). Briefly, aliquots of the reaction mixture were taken at appropriate time intervals, diluted with 0.15 M NaCl, and centrifuged at 2000 rpm for 10 min to separate the RBCs. The percentage haemolysis was determined by measuring the absorbance of the supernatant at 540 nm and compared with that of complete haemolysis by treating the same RBC suspension with distilled water. In the case of anti-haemolysis experiments, curcumin and its analogues, and/or α-tocopherol dissolved in dimethyl sulfoxide (DMSO) was added and incubated before addition of AAPH. The final concentration of DMSO was 0.1% (v/v) and did not interfere with the determination. Every experiment was repeated three times and the results were reproducible within 10% deviation.

3. Results and discussion

3.1. AAPH-induced RBC haemolysis

Thermal decomposition of AAPH in the aqueous dispersion of RBCs produces an initiating radical ($R^{\cdot}$) which can attack the polyunsaturated lipids (LH) in RBC membranes to induce lipid peroxidation [Eqs. (1)–(6)]. The initiation rate of AAPH at 37 °C in aqueous dispersions has been determined to be 1.3×10^{-6} [AAPH]/s (Bowry & Stocker, 1993). Since the lipid peroxidation is a free radical chain reaction and one initiating radical could induce up to fifty propagation reactions (Bowry & Stocker, 1993; Hou et al., 2004a, 2004b), the RBC membrane is quickly damaged, leading to haemolysis. On the other hand, if antioxidants (AHs), such as vitamin E, vitamin C and curcumin, are present or added to RBCs they would react with the chain-propagating peroxyl radicals to stop the peroxidation [Eq. (7)], and hence inhibit haemolysis.

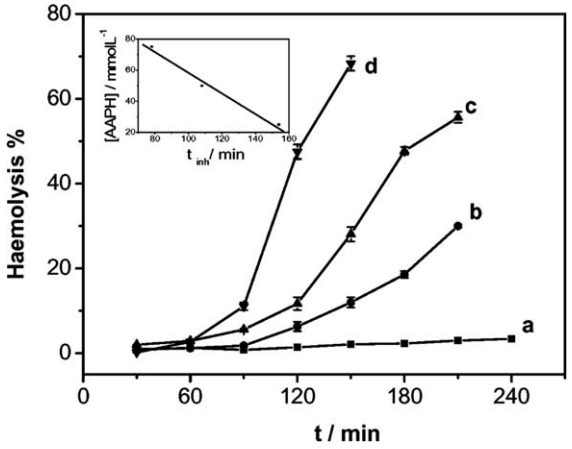


Fig. 1. AAPH-induced haemolysis of 5% human RBCs in PBS (0.15 M, pH 7.4) in an aerobic atmosphere at 37 °C. The initial concentrations of AAPH were: (a) 0; (b) 25 mM; (c) 50 mM; (d) 75 mM. The inset shows the relationship between the inhibition time, t_{inh} and the initial concentration of AAPH. Data are expressed as the means of three RBC samples.

$$\text{Initiation}: \quad \mathrm{R{-}N{=}N{-}R} \rightarrow 2\mathrm{R}^{\bullet} + \mathrm{N_2} \quad (1)$$

$$\mathrm{R}^{\bullet} + \mathrm{O_2} \rightarrow \mathrm{ROO}^{\bullet} \quad (2)$$

$$\mathrm{ROO}^{\bullet} + \mathrm{LH} \rightarrow \mathrm{ROOH} + \mathrm{L}^{\bullet} \quad (3)$$

$$\text{Propagation}: \quad \mathrm{L}^{\bullet} + \mathrm{O_2} \rightarrow \mathrm{LOO}^{\bullet} \quad (4)$$

$$\mathrm{LOO}^{\bullet} + \mathrm{LH} \rightarrow \mathrm{LOOH} + \mathrm{L}^{\bullet} \quad (5)$$

$$\text{Termination}: \quad \mathrm{LOO}^{\bullet} + \mathrm{LOO}^{\bullet} \rightarrow \text{molecular products} \quad (6)$$

$$\text{Antioxidation}: \quad \mathrm{LOO}^{\bullet} + \mathrm{AH} \rightarrow \mathrm{LOOH} + \mathrm{A}^{\bullet} \quad (7)$$

Fig. 1 shows the AAPH-induced RBC haemolysis in an aerobic atmosphere. In the absence of AAPH, the RBCs were stable and little haemolysis took place within 4 h. Addition of AAPH induced, after an inhibition period, fast haemolysis. Since no inhibition period was observed in micellar model systems after the addition of AAPH (Fang et al., 2002; Zhou et al., 2005a, 2005b), this inhibited haemolysis must stem from the action of native bioantioxidants, e.g., vitamin E and/or ubiquinol-10, present in biomembranes (Hou et al., 2004b). We have observed similar inhibited peroxidation in AAPH-induced peroxidation of human erythrocyte ghosts and low-density lipoprotein (Hou et al., 2004a, 2004b; Liu et al., 2000; Zhou et al., 2004). It is seen from Fig. 1 that the rate of haemolysis and the inhibition time are correlated dose-dependently with the concentration of AAPH. The inhibition time was 106 ± 3 min when the concentration of AAPH was 50 mM.

3.2. Inhibition of RBC haemolysis by curcumin and its analogues

Addition of curcumin and its analogues (ArOHs) to the 5% RBC suspension significantly increased the intrinsic inhibition time of the RBCs. The inhibition time produced by ArOHs depended on the concentration of the ArOH, as illustrated in Fig. 2 and on the specific ArOH used as shown in Fig. 3. When initiated with 50 mM of AAPH, the inhibition times produced by 10 μM, concentrations of compounds **1**–**7** were 166 ± 2, 165 ± 2, 153 ± 3, 132 ± 4, 217 ± 4, 229 ± 6 and 163 ± 3 min, respectively. These correspond to the additional or effective inhibition times, t_{eff}, produced by these antioxidants, being 60, 59, 47, 26, 111, 123 and 57 min, respectively. It is clearly seen that the

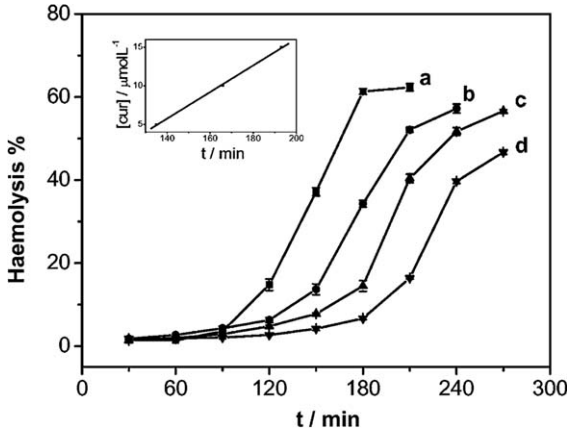


Fig. 2. Inhibition of AAPH-induced haemolysis of human RBCs by curcumin (**1**). The experimental conditions were the same as described in the legend of Fig. 1 with $[AAPH]_0 = 50$ mM. The initial concentrations of **1** were: (a) 0; (b) 5 μM; (c) 10 μM; (d) 15 μM. The inset shows the relationship between the inhibition time, t_{inh}, and the initial concentration of **1**. Data are expressed as the means of three RBC samples.

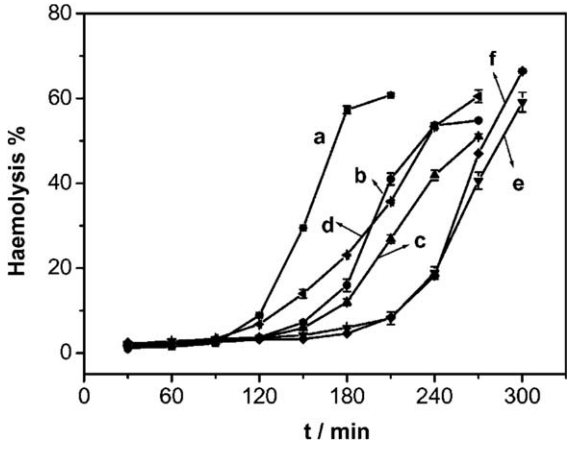


Fig. 3. Inhibition of AAPH-induced haemolysis of human RBCs by curcumin and its analogues (ArOHs). The experimental conditions were the same as described in the legend of Fig. 2 with $[ArOH]_0 = 10$ μM. (a) Native RBCs; (b) inhibited with **1**; (c) inhibited with **2**; (d) inhibited with **3**; (e) inhibited with **5**; (f) inhibited with **6**. Lines for **4** and **7** are not shown for clarity. Data are expressed as the means of three RBC samples.

activity of these compounds depends significantly on the number of phenolic groups in the molecules. Compound **6**, which possesses four phenolic groups, is the most active one ($t_{eff} = 123$ min), followed by compound **5**, which possesses three phenolic groups ($t_{eff} = 111$ min). Compounds **1**, **2** and **7**, which possess two phenolic groups, exhibit similar activities ($t_{eff} \sim 60$ min). Compound **3**, with one phenolic group, is the least active one ($t_{eff} = 47$ min) except **4** for which bears no phenolic group. The weak but appreciable activity of **4** ($t_{eff} = 26$ min) will be discussed below.

3.3. The antioxidant mechanism

The antioxidant mechanisms of curcumin have recently been a focus of interest of free radical chemists and biologists (Barclay et al., 2000; Jovanovic, Steenken, Boone, & Simic, 1999; Priyadarsini et al., 2003; Sun, Zhang, Chen, & Liu, 2002; Zheng et al., 1997). The central argument was whether the phenolic hydrogen or the central methylenic hydrogen in the heptadienone moiety is responsible for its antioxidant activity. Jovanovic et al. (1999) studied (by laser flash photolysis and pulse radiolysis) the reaction of curcumin with methyl and *tert*-butoxyl radicals, and concluded that, in acidic and neutral aqueous and acetonitrile solutions, curcumin is a superb H-atom donor by donating the H-atom from the central methylenic group rather than from the phenolic group (Jovanovic et al., 1999). On the other hand, Barclay and collaborators compared the antioxidant activities of curcumin (**1**) and dimethylcurcumin (**4**) against free radical-initiated peroxidation of styrene in chlorobenzene solution and concluded that curcumin is a classical phenolic chain-breaking antioxidant, donating H-atoms from the phenolic groups (Barclay et al., 2000). Recently, Priyadarsini et al. (2003) also compared the antioxidant activity of **1** and **4** against radiation-induced lipid peroxidation of rat liver microsomes and the free radical-scavenging activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH), and concluded that the phenolic group plays a major role in the activity of curcumin. Theoretical calculations, by density functional theory (DFT), demonstrated that the enol form of curcumin is significantly more stable than the diketo form and that the bond dissociation enthalpy (BDE) of the phenolic O–H bond is significantly lower than the BDE of the central C–H bond, suggesting that the H-atom abstraction should take place in the phenolic group (Priyadarsini et al., 2003; Sun et al., 2002; Wright, 2002). It was also pointed out that the relative contribution of the phenolic group and the central methylenic group on the antioxidant activity depends on the activity of the attacking radical and the reaction medium (Wright, 2002; Zheng et al., 1997).

The present work indicates that the effective inhibition period, t_{eff}, in the inhibition of RBC haemolysis produced by these curcumin analogues is correlated with the number of the phenolic groups. This is in accordance with the result reported by (Barclay et al., 2000) that the stoichiometric factor, n (the number of peroxyl radicals trapped per molecule of the antioxidant), is proportional to the number of the phenolic groups in curcumin analogues in their inhibition of styrene peroxidation. Therefore, it is evident that the anti-haemolysis effect of curcumin and its analogues is due to their antioxidant effect on free radical-initiated peroxidation of RBC membranes, and that the phenolic groups in the molecules play a crucial role in their antioxidation and anti-haemolysis activity.

In this context, the appreciable anti-haemolysis activity of compound **4** which bears no phenolic group is interesting. This compound was reported to be inactive on inhibition of styrene peroxidation (Barclay et al., 2000), hence precluding the responsibility of H-atom donation from the central methylenic group for the anti-haemolysis effect. Similar different antioxidative behaviour of **4** in chemical and biological systems has been recently reported (Priyadarsini et al., 2003). The efficiencies of inhibiting radiation-induced lipid peroxidation of rat liver microsomes by curcumin (**1**) and dimethylcurcumin (**4**) were reported to be 82% and 24%, respectively, while the activity of **4** on scavenging DPPH radical in acetonitrile solution was negligible (~1800 times less than **1**) (Priyadarsini et al., 2003). Therefore, there must be some biologically active constituents present in biomembranes that help **4** to exhibit anti-haemolysis activity. Indeed, it has been reported previously (Zheng et al., 1997) that schisandrin, which is a polymethoxylated naturally-occurring aromatic compound, could effectively inhibit free radical-induced haemolysis of mouse erythrocytes, and that addition of chlorhexidine, which can inhibit the *O*-demethylase activity (Flemming & Jensen, 1974; Ribbons & Harrison, 1971), almost completely suppressed the anti-haemolysis activity of schisandrin. Therefore, it seems likely that the *O*-demethylase present in the cells (Flemming & Jensen, 1974) plays a crucial role in the anti-haemolysis of **4**. That is, the enzyme catalyses the demethylation of **4** and produces the corresponding phenolic compound, *e.g.*, compound **3**, which is the active metabolic product responsible for the anti-haemolysis effect.

3.4. Anti-haemolysis effect of curcumin and its analogues in the presence of external α-tocopherol

It is well-known that α-tocopherol (TOH), the most abundant and active form of vitamin E, is a principal lipid-soluble chain-breaking antioxidant in plasma and RBCs (Burton & Ingold, 1986). Its synergistic antioxidative effect with other antioxidants, such as L-ascorbic acid (vitamin C) (Liu, Liu, & Han, 1988), green tea polyphenols (Zhou et al., 2000, 2005b), and resveratrol

(Fang et al., 2002) has been well documented and proved to be due to the reduction of α-tocopheroxyl radical ($TO^{\cdot}$) by the co-existent antioxidant (ArOH) to regenerate TOH [Eq. (8)].

$$TO^{\bullet} + ArOH \rightarrow TOH + ArO^{\bullet} \quad (8)$$

Therefore, it is desirable to see if curcumin and its analogues possess similar synergistic anti-haemolysis effect with TOH. A representative result is shown in Fig. 4. It can be seen that addition of TOH (25.5 μM) produced an additional inhibition time of 39 min, and addition of curcumin (**1**) (10 μM) produced an additional inhibition time of 59 min. When the two antioxidants were used together, the additional inhibition time was 95 min; that is approximately the sum of the inhibition times when **1** and TOH were used individually. Other curcumin analogues gave similar results (figures not shown). That is, the two antioxidants acted independently and no synergistic interaction was present between curcumin and TOH in the anti-haemolysis action. This is understandable because the oxidation potential of curcumin (0.77 V at pH 7) is considerably higher than that of α-tocopherol (0.48 V at pH 7) (Jovanovic et al., 1999), rendering the α-tocopherol regeneration reaction [Eq. (8)] by curcumin an unfavourable process.

It is also seen from Figs. 3 and 4 that curcumin and its analogues are significantly more effective than α-tocopherol in their anti-haemolysis effects. The t_{eff} produced by 10 mM of curcumin (59 min) is ca. four times longer than the t_{eff} produced by the same amount of α-tocopherol (ca. ~ 15 min). Compounds **5** and **6**, which bear an *ortho*-hydroxyphenolic functionality, are even more active. Obviously, this is due to, not only the number of phenolic groups, but also the high reactivity of *ortho*-methyoxyphenolic and *ortho*-hydroxyphenolic functionalities. It has been proved that the *ortho*-methoxyl group can form an intramolecular hydrogen bond with the phenolic hydrogen, making the H-atom abstraction from the *ortho*-methoxyphenols surprisingly easy (de Heer, Mulder, Korth, Ingold, & Lusztyk, 2000). It was also known that the *ortho*-hydroxyl substitution on phenol would make the oxidation intermediate, *ortho*-hydroxyphenoxyl radical, more stable due to the intramolecular hydrogen bonding interaction as reported recently from both experiment (Foti & Ruberto, 2001) and theoretical calculations (Wright, Johnson, & Dilabio, 2001). The theoretical calculation showed that the intramolecular H-bond in *ortho*-OH phenoxyl radical is ca. 4 kcal/mol stronger than that in the parent molecule catechol and the BDE of catechol is 9.1 kcal/mol lower than that of phenol (Wright et al., 2001). We have recently found a significantly higher antioxidant activity of molecules bearing *ortho*-diphenoxyl functionality in flavonols (Hou et al., 2004a, 2004b; Zhou et al., 2005a) and resveratrol analogues (Cai et al., 2003; Fang et al., 2002).

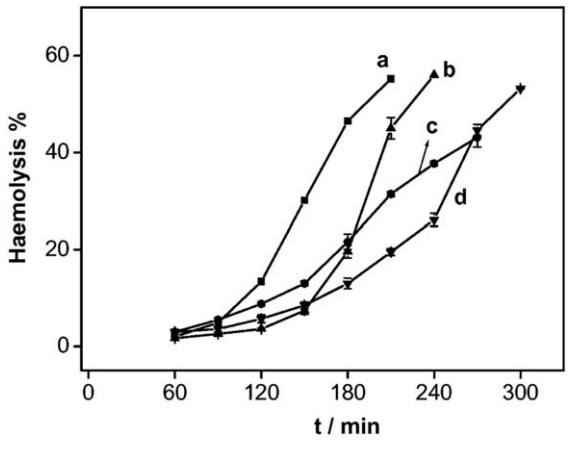


Fig. 4. Inhibition of AAPH-induced haemolysis of human RBCs by curcumin (**1**) and α-tocopherol (TOH). The experimental conditions were the same as described in the legend of Fig. 2. (a) native RBCs; (b) inhibited with **1** (10 μM); (c) inhibited with TOH (25.5 μM); (d) inhibited with **1** (10 μM) and TOH (25.5 μM). Data are expressed as the means of three RBCs samples.

In conclusion, curcumin and its analogues **2**–**7** are effective antioxidants which can protect human red blood cells from free radical- induced oxidative haemolysis and the H-atom abstraction from the phenolic group is responsible for the activity. The observation that the compounds bearing *ortho*-diphenoxyl functionality exhibit markedly higher anti-haemolysis activities than those bearing no such functionality gives us useful information for antioxidant drug design.

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