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Including 4-Hydroxyquinoline Derivatives into β-Cyclodextrin to Form Complexes Affects Their Antioxidative Effect on Free-Radical-Induced Hemolysis of Human Erythrocytes

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Full Paper

A series of β-cyclodextrin inclusion complexes of 4-hydroxyquinoline derivatives was designed and their activities on free-radical-induced hemolysis were studied. The hemolysis of human erythrocytes was initiated by 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH). A novel distributive status was designed: 7-chloro-4-hydroxyquinoline-3-carboxylic acid (CA), 7-fluoro-4-hydroxyquinoline-3-carboxylic acid (FA), 7-chloro-4-hydroxyquinoline (CQ) and 7-fluoro-4-hydroxy quinoline (FQ) were included into β-cyclodextrin to form host-guest complexes, CACD, FACD, CQCD and FQCD, respectively. The hemolysis process was expressed mathematically by Boltzmann equation. Three complexes acted as concentration-dependent antioxidants, and the order of *concentration-sensitivity* was CQCD > FACD > FQCD. CACD did not show activity. The order of 50% inhibitory concentration (IC_{50}) was FQCD < FACD < CQCD, which was different from that dissolved in dimethyl sulfoxide (DMSO) remarkably. FQCD may be a candidate for a novel antitumor drug.

1 Introduction

The association of free radical damage with many disease states was well documented. The essential consideration was to synthesize compounds and/or extract natural ones to detect their antioxidative activities for scavenging the initiating and/or propagating free radicals, finally develop the clinic usage [1–4]. We have designed a series of 4-hydroxyquinoline derivatives and detected their antioxidative effects on 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH) induced hemolysis of human erythrocytes. It was found that the antioxidative/prooxidative effect of these compounds was not only related to the substituent of quinoline, but their distributive status, dissolved in dimethyl sulfoxide (DMSO) or packaged in dipalmitoyl phosphatidylcholine (DPPC) vesicles, affected the activities remarkably [5]. This background motivated us to design a novel distributive status: including 7-chloro-4-hydroxyquinoline-3-carboxylic acid (CA), 7-fluoro-4-hydroxyquinoline-3-carboxylic acid (FA), 7-chloro-4-hydroxyquinoline (CQ) and 7-fluoro-4-hydroxyquinoline (FQ) into β-cyclodextrin (CD) to form host-guest complexes, CACD, FACD, CQCD and FQCD, respectively, and to detect the antioxidative effects of these complexes on AAPH-induced hemolysis.

The erythrocyte membrane contains abundant polyunsaturated fatty acids that are very susceptible to be peroxidized by free radicals. The decomposition of the water-soluble azo compound, AAPH, at 37 °C generates free radicals concentration-dependent that could attack the erythrocyte membrane to induce hemolysis eventually. Therefore, the hemolysis induced by AAPH provides a good approach to research the free-radical-induced mem-

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Key words: free radical; antioxidant; erythrocyte; hemolysis; β-cyclodextrin; 4-hydroxyquinoline derivatives; structure-activity relationship

Abbreviations: PBS, phosphate-buffered saline; AAPH, 2,2′-azobis(2-amidino propane hydrochloride); DMSO, dimethyl sulfoxide; CD, β-cyclodextrin; ArOH, 4-hydroxyquinoline derivatives; CD-ArOH, β-cyclodextrin complexes of 4-hydroxy quinoline derivatives; CA, 7-chloro-4-hydroxyquinoline-3-carboxylic acid; FA, 7-fluoro-4-hydroxyquinoline-3-carboxylic acid; CQ, 7-chloro-4-hydroxyquinoline; FQ, 7-fluoro-4-hydroxyquinoline; CACD, β-cyclodextrin complex including 7-chloro-4-hydroxyquinoline-3-carboxylic acid; FACD, β-cyclodextrin complex including 7-fluoro-4-hydroxyquinoline-3-carboxylic acid; CQCD, β-cyclodextrin complex including 7-chloro-4-hydroxyquinoline; FQCD, β-cyclodextrin complex including 7-fluoro-4-hydroxyquinoline; t_0, the lag time of 50% hemolysis; IC_{50}, inhibitory concentration for 50% inhibition of hemolysis.

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brane damage and evaluate the antioxidative activity of the compounds *in vitro* [6].

Presented here is the study of the antioxidative effects of CACD, FACD, CQCD and FQCD on AAPH-induced hemolysis of human erythrocytes. The hemolysis process is expressed mathematically by Boltzmann equation. The inhibitory concentration, IC_{50}, is discussed as well.

2 Materials and Methods

2.1 Materials

AAPH, DMSO and β-cyclodextrin were purchased from Aldrich and used as received. Human erythrocytes were collected from a healthy adult donor by venipunture into heparin. Erythrocytes were washed three times with phosphate-buffered saline (PBS: NaCl, 150 mM; Na_2HPO_4, 8.1 mM; NaH_2PO_4, 1.9 mM; pH 7.4) [7] to remove the plasma, and centrifuged at $1700 \times g$ for exact 10 min to obtain compact erythrocytes. Then the compact erythrocytes were added to PBS (pH 7.4) to form 3.0% suspension (v/v) for the experimental use.

CA, FA, CQ and FQ were synthesized according to Ref. [8]. The β-cyclodextrin complexes of the 4-hydroxyquinoline derivatives were synthesized by dry mixing method [9, 10] (β-cyclodextrin/4-hydroxyquinoline derivatives = 1 (molar ratio)) and characterized by element analysis and H,H-COSY NMR spectra (not shown here), in which a cross peak between H1 of β-cyclodextrin and H2 of quinoline demonstrated that the structures of the complexes were according to the following scheme (the trapezoid indicated the framework of β-cyclodextrin) [11] .

2.2 Determination of Erythrocyte Hemolysis

The experimental process of erythrocytes hemolysis was as described in the literature [5, 6, 12, 13, 14]. CD-ArOHs were dissolved in DMSO, and added to the 3.0% suspension of human erythrocytes in PBS (pH 7.4) and incubated at 37 °C (± 0.1 °C) for 5 min. An AAPH/PBS solution was injected into the above incubation mixture to initiate hemolysis. In every experiment, the final concentration of AAPH in the incubation mixture was kept at 40 mM. Aliquots of the incubation mixture were taken at appropriate time intervals and centrifuged to remove erythrocytes and obtain the supernatant. The percentage of hemolysis ($(A/B) \times 100$) was determined by measuring the absorbance of the supernatant at 540 nm (A) and compared with that of complete hemolysis (B). The concentration of CD-ArOH for 50% inhibition of AAPH-induced hemolysis, IC_{50}, was determined graphically by using a regression method with the final concentrations of CD-ArOH ranging from 0 μM, as a control, to 67.5 μM, while 150 min was appropriate as incubation period for the determination of IC_{50} in this case [15]. Every experiment was repeated at least three times for the reproducibility within 10% experimental error. Experimental data were under one-way ANOVA statistics by Origin Sever software (version 5.0).

3. Results and Discussion

3.1 Antioxidative Activities of CACD, FACD, CQCD and FQCD

Figure 1, Figure 2, Figure 3 and Figure 4 show the variation of hemolysis percentage with the increase of incubation time. The final concentration of AAPH was selected to be 40 mM since the lag time of hemolysis was correlated linearly with the concentration of AAPH [5], and the final concentrations of CACD, FACD, CQCD and FQCD ranged from 0 μM, as the control, to 67.5 μM with the interval of 15.0 μM, respectively.

It can be found from the line a in the above figures that the hemolysis was still lagged in the absence of any CD-ArOH, indicating that the endogenous antioxidants in the erythrocyte, *i.e.* α-tocopherol, catalase, superoxide dismutase, and glutathione, can trap the initiating and/or propagating radicals to protect the erythrocytes against free-radical-induced hemolysis [6]. After depletion of all the endogenous antioxidants, hemolysis starts rapidly. Finally, the hemolysis becomes slow at the end of the reaction period. The addition

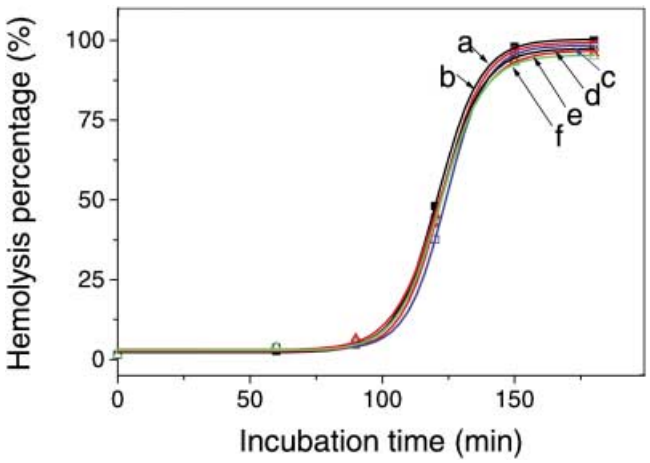
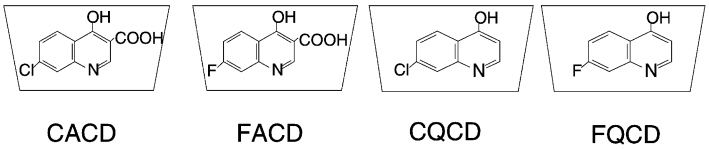


Figure 1. Hemolysis curves of human erythrocytes in the presence of CACD with various concentrations. (a) 3.0% erythrocyte + 40 mM AAPH; (b) (a) + 7.50 μM CACD; (c) (a) + 22.5 μM CACD; (d) (a) + 37.5 μM CACD; (e) (a) + 52.5 μM CACD; (f) (a) + 67.5 μM CACD.



CACD FACD CQCD FQCD

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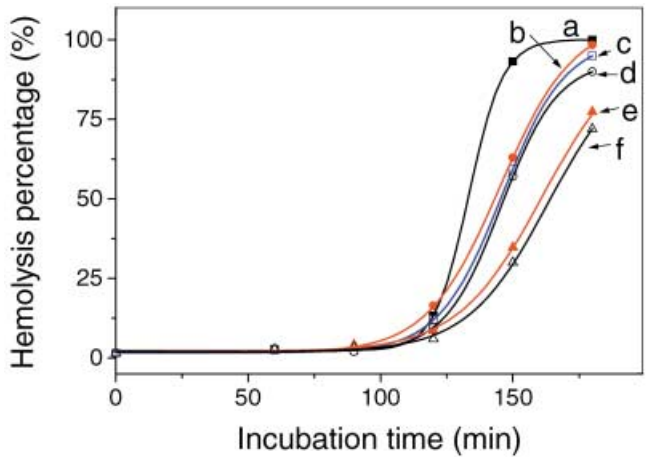

Figure 2. Hemolysis curves of human erythrocytes in the presence of FACD with various concentrations. (a) 3.0% erythrocyte + 40 mM AAPH; (b) (a) + 7.50 μM FACD; (c) (a) + 22.5 μM FACD; (d) (a) + 37.5 μM FACD; (e) (a) + 52.5 μM FACD; (f) (a) + 67.5 μM FACD.

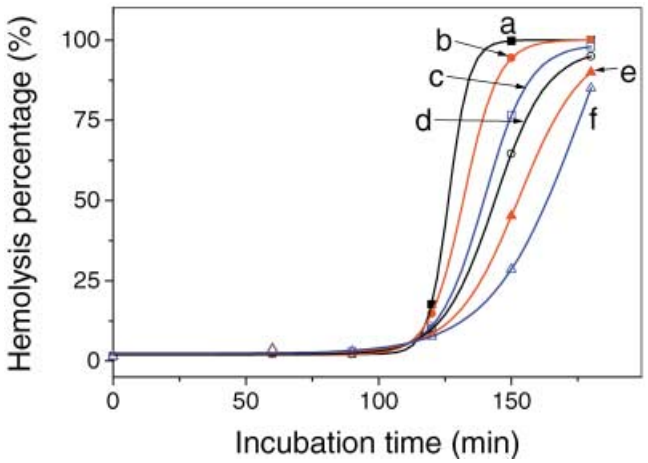

Figure 3. Hemolysis curves of human erythrocytes in the presence of CQCD with various concentrations. (a) 3.0% erythrocyte + 40 mM AAPH; (b) (a) + 7.50 μM CQCD; (c) (a) + 22.5 μM CQCD; (d) (a) + 37.5 μM CQCD; (e) (a) + 52.5 μM CQCD; (f) (a) + 67.5 μM CQCD.

of CD-ArOH affected the slope in the period of hemolysis, indicating that CD-ArOH can protect erythrocytes against AAPH-induced hemolysis. The variation of hemolysis percentage with the increase of incubation time can be expressed mathematically by Boltzmann equation:

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

where H refers to hemolysis percentage and t stands for the incubation time. The constants, $A_{initial}$ and A_{final} indicate the hemolysis percentage at the beginning and the end of reaction, respectively. The coefficient, t_0, reveals the time of

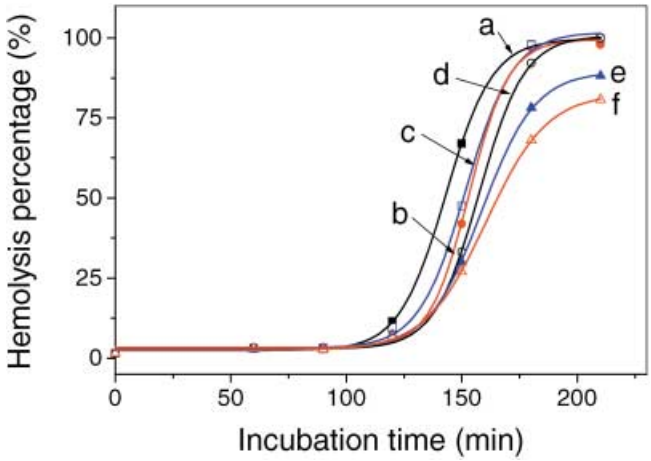

Figure 4. Hemolysis curves of human erythrocytes in the presence of FQCD with various concentrations. (a) 3.0% erythrocyte + 40 mM AAPH; (b) (a) + 7.50 μM FQCD; (c) (a) + 22.5 μM FQCD; (d) (a) + 37.5 μM FQCD; (e) (a) + 52.5 μM FQCD; (f) (a) + 67.5 μM FQCD.

hemolysis reaching 50%, involving the lag time and half time of hemolysis. Hence, t_0, can be used to evaluate the protective effect of CD-ArOH on AAPH-induced hemolysis. All the hemolysis curves are expressed mathematically by Boltzmann equations, and the constants, $A_{initial}$ and A_{final}, and coefficient, t_0, are collected and listed in Table 1.

As can be seen in Table 1 t_0 is increased by addition of FACD, CQCD and FQCD, respectively, revealing that they play antioxidative role in AAPH-induced hemolysis. However, the addition of CACD cannot prolong t_0 remarkably, demonstrating that CACD is not an antioxidant or prooxidant in above experimental system. In order to clarify the relationship between the antioxidative activities of CD-ArOHs and their concentrations quantitatively, the relationship between t_0 and the concentration of CD-ArOH was illustrated in Figure 5, and regressed linearly by the following equation:

$$t_0 = A \times C + B \quad (2)$$

The coefficients, A, indicating the *concentration-sensitivity* of t_0, are listed in Table 2.

As can be seen from Table 2, the order of *concentration-sensitivity* of CD-ArOH is CQCD > FACD > FQCD, indicating that t_0 increased more rapidly with the increase of the concentration of CQCD than FACD and FQCD. The *concentration-sensitivity* of CACD, ~ 0, indicates that it cannot play any role in AAPH-induced hemolysis.

We have discussed the antioxidative mechanism of 4-hydroxyquinoline derivatives [5] as the following reactions:

initiation: $RN{=}NR \longrightarrow 2\,R^{\bullet} + N_2$ (3)

$R^{\bullet} + O_2 \longrightarrow ROO^{\bullet}$ (4)

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Table 1. Constants, $A_{initial}$ and A_{final}, and coefficients, t_0 and dt, of various hemolysis curves in the presence and absence of CD-ArOHs with various final concentrations [a)]

CD-ArOH	*concentration (μM)*	t_0	dt	$A_{initial}$	A_{final}
CACD	0.0	121.0	8.1	2.2	100.4
	7.55	122.6	7.7	2.7	99.4
	22.5	124.2	7.5	3.1	98.5
	37.5	121.9	8.2	3.0	97.5
	52.5	121.6	8.8	3.0	96.8
	67.5	122.0	8.1	2.9	95.4
FACD	0.0	133.3	6.5	2.2	100.0
	7.55	145.1	13.8	1.5	106.1
	22.5	145.9	12.0	1.7	100.4
	37.5	145.6	10.7	1.9	93.5
	52.5	161.3	16.2	2.0	100.0
	67.5	163.2	15.2	1.9	95.2
CQCD	0.0	126.4	3.9	2.1	100.0
	7.55	132.1	6.4	1.9	100.0
	22.5	139.8	8.5	2.2	98.8
	37.5	143.6	9.8	2.0	97.3
	52.5	152.8	12.0	2.5	99.1
	67.5	177.1	17.3	2.3	154.9
FQCD	0.0	143.1	10.1	2.4	99.7
	7.55	153.3	8.6	3.3	99.5
	22.5	151.7	10.2	3.1	101.8
	37.5	157.7	9.7	2.8	100.7
	52.5	158.7	11.5	2.8	89.5
	67.5	160.6	13.2	2.4	82.8

a) The hemolysis curves follow the Boltzmann equation, $H = (A_{initial} - A_{final})/(1 + e^{(t-t_0)/dt}) + A_{final}$ (see text). [erythrocyte] = 3.0% (v/v in PBS), temperature = 37 °C, [AAPH] = 40 mM.

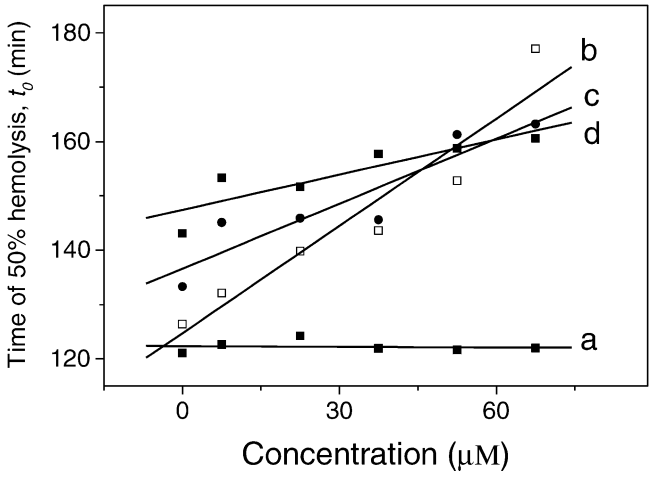

Figure 5. The relationship between the concentrations of CD-ArOHs and lag time of 50% hemolysis, t_0. (a) CACD; (b) CQCD; (c) FACD and (d) FQCD.

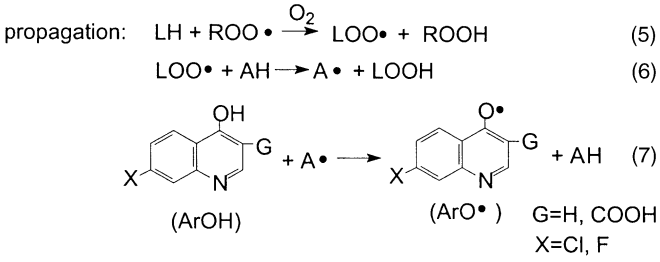

Table 2. Constant, B, and coefficient, A in the relationship between the concentration of CD-ArOHs (C), and the time of 50% hemolysis (t_0) [a)]

CD-ArOH	A	B
CACD	− 0.0033	122.3
FACD	0.40	136.6
CQCD	0.66	124.7
FQCD	0.22	147.4

a) The relationship between the concentration of CD-ArOH (C) and the time of 50% hemolysis (t_0) can be expressed quantitatively by the equation: $t_0 = A \times C + B$ (see text).

in which R• stands for the initial radical generated form the decomposition of AAPH, LOO• refers to the peroxy radical of polyunsaturated fatty acids (LH) from erythrocytes, LOOH stands for peroxide of LH, and AH refers to the endogenous antioxidants in erythrocytes. The antioxidative activity of ArOH was not only related to their structures, but the distributive status of ArOH can also affect their activities remarkably, *i.e.*, FA and CA (G=COOH) accelerated hemolysis if they were distributed in DPPC vesicles. On the contrary, they protected erythrocytes against AAPH-induced hemolysis while dissolved in DMSO directly [5]. After included into β-cyclodextrin, the distributive status of ArOH is varied from a single molecule to a guest-host complex, equilibrium between CD-ArOH and decomposition into ArOH and β-cyclodextrin should be

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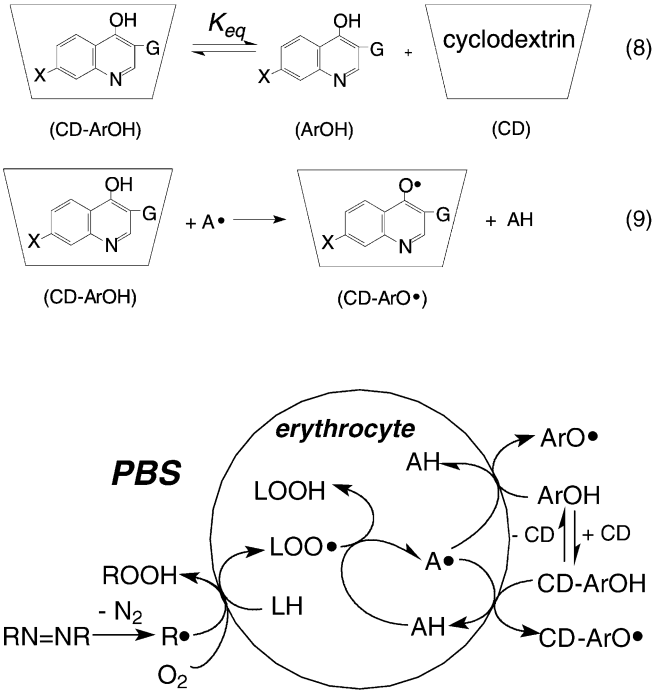


Scheme 1. The antioxidative mechanism of CD-ArOH.

taken into account in this case (Eq. 8). Meanwhile, CD-ArOH may also protect erythrocyte against AAPH-induced hemolysis directly by repairing endogenous antioxidant radical as Eq. 9 shows. Therefore, the antioxidative activities of CD-ArOH are the combined results of Eqs. 7, 8 and 9.

The antioxidative mechanism of CD-ArOH can be illustrated by Scheme 1.

3.2 The IC_{50} of CACD, FACD, CQCD and FQCD

The determination of 50% inhibitory concentration (IC_{50}) was essential for the comparison of the antioxidative activity in view of chemistry. Vinson and cooperators provided a simple way to obtain IC_{50} [15]. In brief, the antioxidative percentage after a certain reaction time was determined while various concentration of antioxidants were used, then the relationship between the concentrations and corresponding antioxidative percentage was shown graphically. The IC_{50} was the concentration point when inhibitory percentage was 50%. We herein choose 150 min as the incubation period to compare the inhibitory percentage. The corresponding *inhibitory percentage* can be obtained by (100%-hemolysis percentage). The relationship between the *inhibitory percentage* and the concentration is regressed linearly and shown in Figure 6. Therefore, the IC_{50} can be obtained from the cross points of the line b, c, d in Figure 6 and the horizontal dot line [15], and is listed in Table 3.

As can be seen in Table 3, the order of IC_{50} is FQCD < FACD < CQCD, indicating that FQCD is the best antioxidant among these CD-ArOHs. The IC_{50} of CACD is not obtained, demonstrating that it cannot play any role in this experimental system. By comparing the IC_{50} of CD-ArOH with that of ArOH dissolved in DMSO, one can find that the IC_{50} is larger than in DMSO, respectively. This may be due to the molecular volume being so large to make them be '*packaged tightly*' in β-cyclodextrin, resulting in a slow decomposition reaction (see Eq. 8), therefore, these compounds '*packaged tightly*' in β-cyclodextrin may be released slowly to the aqueous phase to play antioxidative role in AAPH-induced hemolysis that occurred particularly between the aqueous phase and lipid phase of erythrocytes. This may be a novel form of the usage of these drugs.

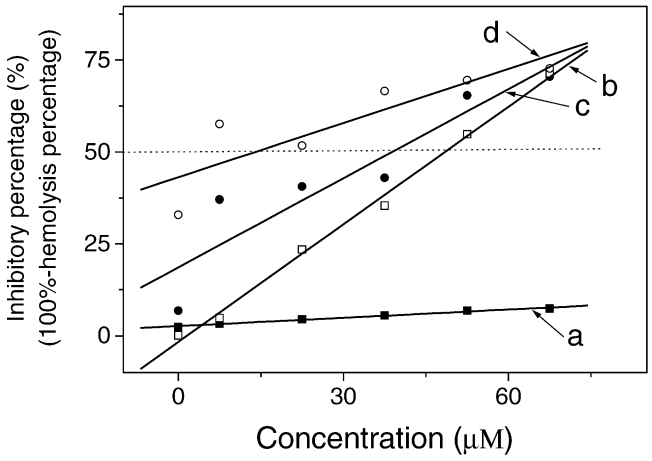
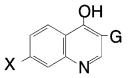


Figure 6. The relationship between the concentrations of CD-ArOHs and inhibitory percentage (100%-hemolysis) while the incubation period is 150 min. (a) CACD; (b) CQCD; (c) FACD and (d) FQCD.

Table 3. Inhibitory concentrations for 50% inhibition of hemolysis, IC_{50}, while the incubation period is 150 min.

	IC_{50} (μM)			
	included into β-cyclodextrin		dissolved in DMSO [a]	
X=Cl; G=COOH	CACD	–[b]	CA	12.0
X=F; G=COOH	FACD	38.8	FA	5.7
X=Cl; G=H	CQCD	48.4	CQ	20.0
X=F; G=H	FQCD	14.0	FQ	11.2

a Cited from Ref. 5;
b Line a in Figure 6 did not reach 50% inhibitory line, so IC_{50} of CACD cannot be obtained.

4 Conclusion

This work revealed that including CA, FA, CQ and FQ into β-cyclodextrin to form complexes, CACD, FACD, CQCD

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and FACD, respectively, changed their antioxidative activities in AAPH-induced hemolysis. The order of *concentration- sensitivity* is FQCD < FACD < CQCD. In view of the order of IC_{50}, FQCD < FACD< CQCD, the antioxidative activity is FQCD >FACD > CACD. CACD, however, did not show activity. In particular, FQCD may be a candidate for a novel form of usage of the antitumor drug, 7-fluoro-4-hydroxyquinoline.

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