Selected Paper

Kinetic Study of Radical-Scavenging and Vitamin E-Regenerating Actions of Edaravone (3-Methyl-1-phenyl-2-pyrazolin-5-one)

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A kinetic study was performed for edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) in order to clarify the mechanism of its free-radical-scavenging and vitamin E-regenerating actions. The second-order rate constants for the radical-scavenging reaction of edaravone were measured in several organic solvents and in a water/methanol mixed solvent at various pH. The keto–enol tautomerism and the acid–base dissociation equilibrium of edaravone produce keto, enol, and anion forms in solutions, and their contributions and activities varied depending on the properties of the solutions. From the results of NMR and kinetic studies, it has been clarified that the keto–enol tautomerism of edaravone actually exists, and the keto form has larger radical-scavenging activity than the enol form. Furthermore, the pH dependence of the rate constants suggests that the anion form produced by the acid–base dissociation equilibrium of edaravone has the highest radical-scavenging activity in the keto, enol, and anion forms.

Edaravone (2,4-dihydro-5-methyl-2-phenyl-3*H*-pyrazol-3-one or 3-methyl-1-phenyl-2-pyrazolin-5-one) (Fig. 1) is known as a brain-protecting agent that is used clinically to treat acute ischemic stroke, and its pharmacological effects have attracted much attention.[1–16] One of the major factors involved in ischemic brain damage is the production and oxidative action of free-radical species, such as the lipid-peroxyl and hydroxyl radical. The hydroxyl radical is considered to be the most reactive active-oxygen species (AOS), which are generated during ischemia. The pharmacological function of edaravone in vivo is thought to be mainly based on its free-radical scavenging from experimental evidences in the previous reports.[2–16] Many medical and pharmacological effects of edaravone to oxidative stresses have also been reported, for example, inhibitions of lipid-peroxidation,[6] prevention of liver-injury,[7,8] and radioprotection.[9]

Some chemical approaches to the antioxidant activity of edaravone in vitro have recently been reported.[6,10–16] The structure–activity relationship for lipid-peroxidation inhibition by edaravone was investigated by Watanabe et al.[6,10] Their result indicates the importance of the 4-position protons in the pyrazolin ring of edaravone derivatives for the inhibition of lipid-peroxidation. They proposed that the keto–enol tautomerism for edaravone using its pyrazolin 4-position proton and neighboring carbonyl group produces the enol form, which plays a main role in the antioxidant action versus AOS. Another report suggests that the anionic form produced by the proton-dissociation from the enol form of edaravone at high pH is more reactive than the non-dissociated form.[11] The keto–enol tautomerism and acid–base dissociation equilibrium are considered to play important roles in the antioxidant action of edaravone. These keto, enol, and anion forms of edaravone existing in solutions should have different activities versus AOS, and show different contributions in each experimental (or medical) condition. The relationship of their structures to antioxidant activities is important for a detailed understanding of the medical and pharmacological effects of edaravone.

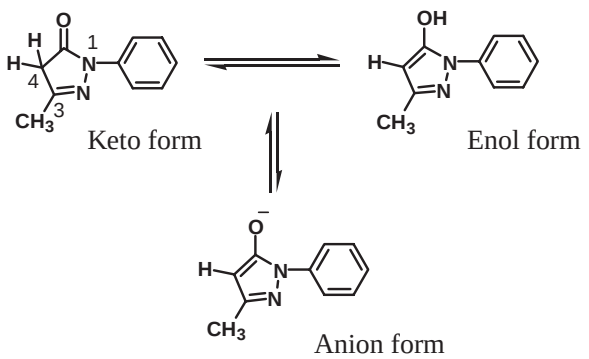

Fig. 1. Molecular structure of edaravone and its keto–enol tautomerism and acid–base equilibrium.

In the present work, a kinetic investigation of the free-radical-scavenging and vitamin E-regeneration actions of edaravone was carried out in several solvents and by varying pH. The second-order rate constants of edaravone for the reactions with the model free radical, aryloxyl (2,6-di-*tert*-butyl-4-(4-methoxyphenyl)phenoxyl, ArO•, Fig. 2a) (Scheme 1), and the regeneration reaction of vitamin E (VE, α-TocH) from α-tocopheroxyl (α-Toc•) (Scheme 2) have been measured using a stopped-flow spectrophotometer in several organic solvents and in a water/methanol mixture at pH 6–8. To investigate the chemical structure of edaravone in solution, ^{1}H NMR spectra for edaravone in chloroform, benzene, acetonitrile, methanol, and DMSO were measured. The kinetic study of the solvent and pH effects on these free-radical-scavenging actions and the NMR study will clarify the importance of the

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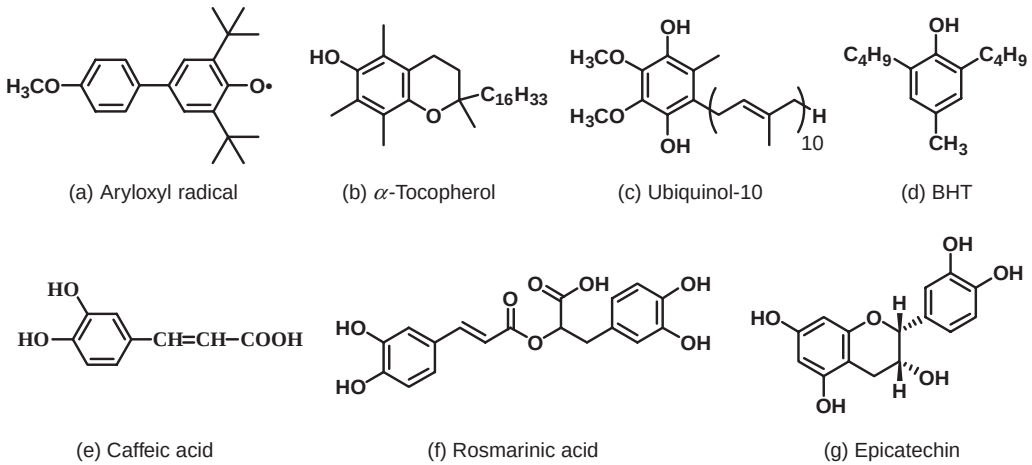

(a) Aryloxyl radical (b) α-Tocopherol (c) Ubiquinol-10 (d) BHT

(e) Caffeic acid (f) Rosmarinic acid (g) Epicatechin

Fig. 2. Molecular structure of aryloxyl radical (ArO•) and some typical antioxidants.

$$\mathrm{ArO}\cdot + \mathrm{Edaravone} \xrightarrow{k_s} \mathrm{ArOH} + \mathrm{Edaravonyl}\cdot$$

Scheme 1.

$$\alpha\text{-Toc}\cdot + \mathrm{Edaravone} \xrightarrow{k_r} \alpha\text{-TocH} + \mathrm{Edaravonyl}\cdot$$

Scheme 2.

contribution of the keto–enol tautomerism and the acid–base dissociation equilibrium of edaravone.

Experimental

Edaravone is a commercially available special-grade reagent from Sigma-Aldrich, and was used as received. α-Tocopherol was obtained from Wako, and was used as received. ArO• was prepared according to a method previously reported.[17] Methanol and ethanol were dried with NaH and purified by distillation. Chloroform, tetrahydrofuran (THF), 1,4-dioxane, acetone, and acetonitrile were obtained from Wako, and purified by distillation. Dimethyl sulfoxide (DMSO) and 1-butanol were obtained from Wako and were used as received. Triton X-100 (TX-100) is an extra-pure grade reagent commercially available from Nacalai Tesque and was used as received. All buffer solutions were prepared using deionized water purified with a Millipore-Q system. The pH of the solutions was adjusted using Na_2HPO_4–KH_2PO_4 buffers (pH 6.0–8.0), whose concentrations were 0.1 mol dm^{-3} (M).[17–20] The concentration of TX-100 in the micelle solutions was kept at 5.0 wt %. The sample solutions were deoxygenated by bubbling nitrogen gas before experiments.

The kinetic data were obtained using a Unisoku RS-450 or RSP-1000 stopped-flow spectrophotometer by mixing equal volumes of an edaravone solution and ArO• or α-Toc• solution at 25 °C.[17–20] The α-Toc• radical was prepared by mixing equal volumes of α-tocopherol and ArO• solutions 2 seconds prior to the mixing of edaravone and α-Toc• solutions using the double-mixing unit of the RSP-1000. The reactions were studied under pseudo-first-order conditions for edaravone, and the absorption decay of ArO• (α-Toc•) was well-characterized as a single exponential decay. The detailed experimental procedures have been reported in previous papers.[17–20]

The pseudo-first-order rate constants (k_{obsd}) for the scavenging reaction of ArO• or α-Toc• by edaravone were estimated with a non-linear least-squares fitting method to a single exponential curve from the decrease in the absorbance at 385 nm of ArO• (Fig. 3) or the absorbance at 420 nm of α-Toc•. The rate constant (k_{obsd}) is given by[17,18]

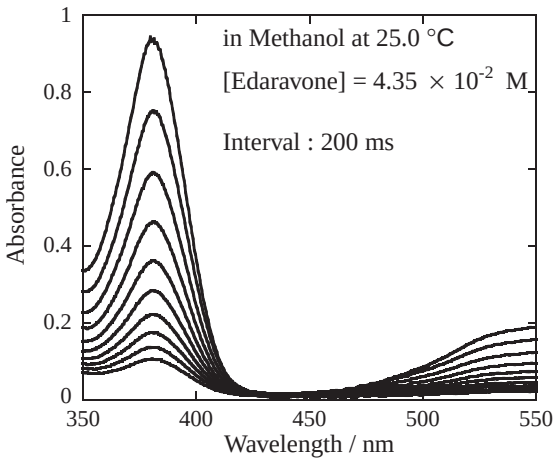

Fig. 3. Time evolution of the absorption spectrum of ArO• in the reaction with edaravone.

$$k_{obsd} = k_0 + k_s[\mathrm{edaravone}], \quad (1)$$

where k_0 is the first-order rate constant for the natural decay of ArO• or α-Toc• in the medium. The second-order rate constant (k_s or k_r) was obtained as a slope of plots of k_{obsd} versus the concentration of edaravone ([edaravone]).

^{1}H NMR measurements of edaravone were performed in five kinds of deuterized solvents (chloroform, acetonitrile, benzene, DMSO, and methanol), using a JEOL 500 MHz NMR spectrometer.

Results and Discussion

(i) The Aryloxyl-Radical-Scavenging Rate Constant (k_s) and the VE-Regeneration Rate Constant (k_r) in Ethanol. The second-order rate constants of edaravone for the ArO• scavenging reaction (k_s) and the VE-regeneration reaction (k_r) obtained in the present study are listed in Table 1, together with those for some antioxidants (Fig. 2) reported in the literatures.[17,19,21,22] The k_s value of edaravone (5.28×10 M^{-1} s^{-1}) was much smaller than those of α-tocopherol (5.12×10^3 M^{-1} s^{-1}) and ubiquinol-10 (5.19×10^3 M^{-1} s^{-1}), and the same

Table 1. Second-Order Rate Constants for the Scavenging Reactions of ArO• (k_s) and α-Tocopheroxyl (k_r) by Edaravone and Some Antioxidants in Ethanol at 25.0 °C

	$k_s/M^{-1}\,s^{-1}$	$k_r/M^{-1}\,s^{-1}$
Edaravone	5.28×10	1.40×10^3
BHT	3.50×10	—
Caffeic acid	5.54×10	2.31×10^2
Rosmarinic acid	9.20×10	9.05×10^2
Epicatechin	1.32×10^2	7.58×10^2 a)
Epigallocatechin gallate	3.36×10^2	2.39×10^4 a)
Rutin	1.42×10	—
Ubiquinol-10	5.19×10^3	2.35×10^5 a)
α-Tocopherol	5.12×10^3	—
Sodium ascorbate	9.98×10^3 a)	2.73×10^6 a)

a) The values in 5:1 (v/v) ethanol–water mixture.

Table 2. Second-Order Rate Constants for the Scavenging Reaction of ArO• with Edaravone (k_s) in Organic Solvents at 25 °C

Solvent	$k_s/M^{-1}\,s^{-1}$	$E_T(25)/kJ\,mol^{-1}$ a)
Acetonitrile	125	193
Acetone	173	177
Chloroform	213	164
THF	229	157
1,4-Dioxane	346	151
Methanol	28.5	232
Ethanol	52.8	217
1-Butanol	85.5	210
DMSO	21.0	188

a) Parameter which takes into account the solvent polarity and hydrogen-bonding ability.[18,23]

order as those for caffeic acid ($k_s = 5.54 \times 10\,M^{-1}\,s^{-1}$) and BHT (butylated hydroxytoluene, 2,6-di-*t*-butyl-4-methylphenol, $k_s = 3.50 \times 10\,M^{-1}\,s^{-1}$). The rate constant of the VE-regeneration reaction for edaravone ($k_r = 1.40 \times 10^3\,M^{-1}\,s^{-1}$) in ethanol is larger than those of caffeic acid, rosmarinic acid, and epicatechin, but 2–3 orders smaller than those of ubiquinol-10 ($2.35 \times 10^5\,M^{-1}\,s^{-1}$) and sodium ascorbate ($2.73 \times 10^6\,M^{-1}\,s^{-1}$). From these results, the free-radical-scavenging activity of edaravone in vitro is considered to be comparable with those of naturally-existing plant-origin polyphenol compounds. These results indicate that edaravone is a good antioxidant for both the free-radical and the VE radical. Edaravone may effectively regenerate VE from α-Toc•, which is produced by the antioxidant reaction of VE versus AOS, such as lipid-peroxyl radicals in membranes.

(ii) Solvent Dependence of the Aryloxyl-Radical-Scavenging Rate Constants (k_s). Table 2 shows the second-order rate constants (k_s) obtained for the scavenging reaction of ArO• by edaravone in methanol, ethanol, 1-butanol, acetone, acetonitrile, THF, 1,4-dioxane, chloroform, and DMSO. The k_s values for edaravone varied from 21.0 to $346\,M^{-1}\,s^{-1}$ depending on the solvent. In low-polarity organic solvents, such as 1,4-dioxane and chloroform, the k_s value is rather larger when compared to those in polar and/or alcoholic solvents. This solvent dependent behavior of the k_s value is similar to

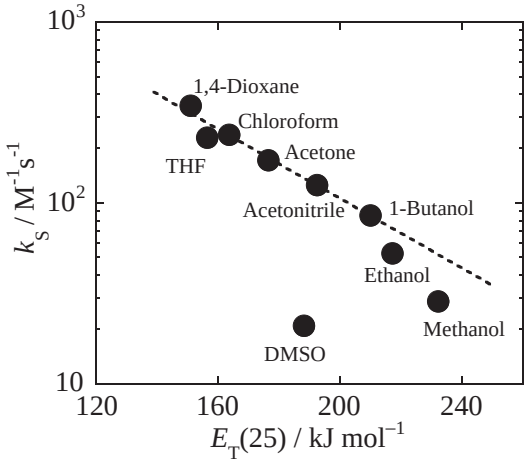

Fig. 4. Semi-logarithm plots of k_s versus $E_T(25)$ for solvents.

that obtained for curcumin reported previously.[18] Figure 4 shows semi-logarithm plots of k_s obtained for edaravone versus $E_T(25)$, which is a parameter that takes into account the solvent polarity and hydrogen-bonding ability.[18,23] These plots for edaravone are separated into three groups of solvents, i.e., (i) acetonitrile, acetone, chloroform, THF, 1,4-dioxane, and 1-butanol, (ii) methanol and ethanol, and (iii) DMSO. The linear relation of logarithms of k_s to $E_T(25)$ for the group of (i) solvents supports that the radical-scavenging reaction by edaravone progresses as a typical hydrogen atom transfer (HAT). However, the different behavior of plots for alcohols and DMSO from those for the other solvents (group (i)) suggests that edaravone has a different way to act in alcohols and DMSO from in the other solvents. Especially, the k_s value in DMSO is very small compared with those in acetonitrile and acetone, which have similar $E_T(25)$ values. Because DMSO and alcohols have strong solvation ability compared with the group (i) solvents, we considered that the solvation to edaravone using hydrogen-bonding affects its antioxidant activity.

To investigate the chemical structure of edaravone in solution, ^{1}H NMR spectra for edaravone in chloroform, benzene, acetonitrile, methanol, and DMSO were measured. The spectrum in chloroform-*d* was assigned reasonably to a keto form of edaravone (Fig. 1) because an NMR line for two protons assigned to the pyrazoline-4-position can be observed around 3.44 ppm. The spectra obtained in benzene-d_6 and acetonitrile-d_3 were similar to that in $CDCl_3$, indicating that edaravone exists as the keto form in these solvents. On the other hand, the NMR spectrum in DMSO was different from that in $CDCl_3$. We could detect only one proton line for the pyrazoline-4-positon proton and this line largely shifted to the low-field side (5.31 ppm) from the corresponding line in $CDCl_3$. Although we could not detect an NMR line for an enol (–OH) proton, we concluded that the spectrum in DMSO came from an enol form of edaravone (Fig. 1). The NMR spectrum obtained in methanol was clearly derived from two species. Two sets of three kinds of phenyl protons (*o*-, *m*-, and *p*-) were observed in 7–8 ppm, and the relative intensities between the two sets are 6:1. This result strongly suggests that the keto and enol forms of edaravone simultaneously exist in methanol. However, because we could not assign the two kinds of NMR lines to the enol and keto forms, we could not determine which

form (keto or enol) is dominant in methanol.

From the results obtained by these kinetic and NMR studies, the solvent effect on k_s can be explained as follows. In group (i) solvents, edaravone exists in the keto form because keto forms are usually more stable than corresponding enol forms. On the other hand, edaravone in DMSO dominantly exists in the enol form, probably because the enol form of edaravone is stabilized by the strong solvation of DMSO to the hydroxy group. The kinetic results in the present study indicate that the keto form has higher activity for HAT than the enol form. The HAT activity of edaravone in these solvents was considered to be based on 4-position protons in the pyrazoline ring. This is consistent with the previous experimental and theoretical studies.[6,12,16] The enol –OH proton seems to have lower activity than that expected from the hydroxy group. One of the reasons for this might be that the enol proton is stabilized by strong binding with solvation.

In alcoholic solvents, the keto and enol forms of edaravone coexist. The reason for this might be that the solvation of alcohols using hydrogen-bonding is weak compared with that of DMSO. Equilibrium between these keto–enol tautomers of edaravone should exist in alcohols. The kinetic results in alcohols suggest that the relative contribution of the enol form increases with increasing $E_T(25)$ value of alcohols. Comparing the k_s plots for alcohols and DMSO in Fig. 4, in alcohols the contribution of the keto form was estimated to be larger than that of the enol form. Taking into account the results obtained by NMR measurements, it was concluded that the ratio of the keto form to the enol form in methanol is 6:1.

As a result, the HAT activity for edaravone versus the free-radical is originated mainly from pyrazolin-4-position protons of the keto form. The keto–enol tautomerism of edaravone actually exists, and the contribution of the enol form to antioxidant action is smaller than that of the keto form.

(iii) pH Dependence of the Aryloxyl-Radical-Scavenging Rate (k_s). In previous works, kinetic studies of the radical-scavenging reaction of vitamin C, flavonoids, and curcumin have been performed in aqueous TX-100 micelle solutions at various pH values using a stopped-flow spectrophotometry.[17–20] The obtained second-order rate constants showed notable pH dependence according to the variation of the mole fraction; also, the activity of some species were produced by the acid–base dissociation equilibrium.

In the present work, the second-order rate constants (k_s) of the scavenging reaction of ArO• by edaravone in a water/methanol (1:1 v/v) mixed solvent were measured with varying the pH. Because of the low solubility of edaravone and ArO• in water, measurements were performed in a water/methanol mixed solvent. Figure 5a shows the plots of the second-order rate constant (k_s) for the scavenging reaction of ArO• by edaravone versus pH in a methanol/water mixed solvent. The observed rate constants increased with an increase of pH from $6.28 \times 10\,M^{-1}\,s^{-1}$ at pH 6 to $1.08 \times 10^2\,M^{-1}\,s^{-1}$ at pH 8. The measurements at pH > 8 and pH < 6 were also tried unsuccessful, because ArO• was unstable in these pH regions. This pH-dependent behavior of k_s is similar to those observed for vitamin C and curcumin previously reported.[17,18] Edaravone has higher activity for radical-scavenging at a higher pH region.

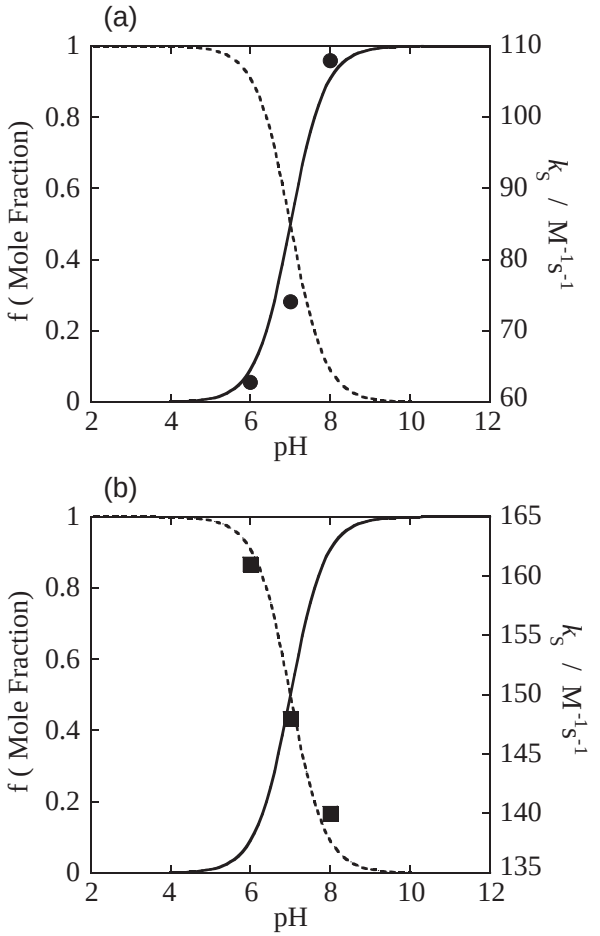

Fig. 5. Plots of second-order rate constant (k_s) for the scavenging reaction of ArO• by edaravone versus pH in (a) methanol/water and (b) TX-100 methanol/water solution. Broken and solid lines show mole-fraction of neutral and dissociated anion forms of edaravone versus pH calculated with assuming $pK_a = 7.0$.

The proton-dissociation equilibrium for edaravone is shown in Fig. 1. The neutral form (EdaH) and dissociated monoanion form (Eda^-) of edaravone will have different rate constants for radical scavenging. The pH dependence of the total reaction rate (k_s^{total}) may be represented as the sum of contributions for both species (Eq. 2):

$$k_s^{total} = k_s^{neu} f(EdaH) + k_s^{anion} f(Eda^-), \quad (2)$$

where k_s^{neu} and k_s^{anion} are second-order rate constants (independent of pH) for EdaH and Eda^-, respectively, and f(EdaH) and $f(Eda^-)$ are pH dependent mole-fractions of EdaH and Eda^-, respectively.[17,18] The broken and solid lines in Fig. 5a show the mole-fractions of neutral and dissociated forms of edaravone versus pH calculated by assuming $pK_a = 7.0$. The k_s^{neu} and k_s^{anion} values were estimated to be 5.81×10 and $1.13 \times 10^2\,M^{-1}\,s^{-1}$ by fitting the observed k_s^{total} values at pH 6–8 to the mole-fraction curve. The pK_a value estimated for edaravone in the present study equals 7.0, as reported previously.[11] The k_s^{neu} value for the neutral form is larger than the k_s value ($28.5\,M^{-1}\,s^{-1}$) measured in methanol. Since the k_s^{anion} value for the anion form is twice k_s^{neu} and the activity of the

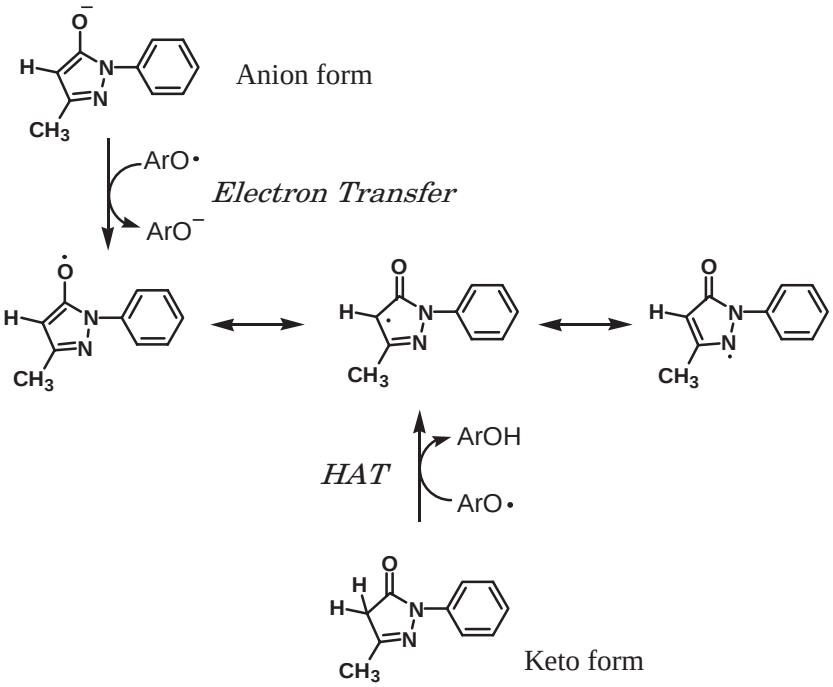

Fig. 6. Antioxidant reaction scheme of edaravone in anion and keto forms.

enol form in organic solvents was low, the free-radical-scavenging reaction for the anion form progresses as an electron-transfer process.

On the other hand, in the 5.0 wt % TX-100 micelle solution of water/methanol (1:1), the k_s value decreases with an increase of pH from $1.61 \times 10^2\ M^{-1}\ s^{-1}$ at pH 6 to $1.40 \times 10^2\ M^{-1}\ s^{-1}$ at pH 8 (Fig. 5b). This pH dependence of k_s is quite different from that in a water/methanol solution. This result may be explained as follows. The ArO• radical exists inside of the micelle because of its lipophilic property. It is difficult for edaravone to have access to ArO• inside of the micelle at high pH because edaravone exists in the anion form and dominantly exists in the water/methanol region outside of the micelle. Ionic species always have difficulty accessing the inside of a micelle because of their poor solubility in the hydrophobic phase.[24] Neutral edaravone at the low pH range easily access the inside of a micelle, and then the scavenging reaction proceeds.

According to the present kinetic study, we concluded that the radical-scavenging activity of edaravone in organic solvents comes from the HAT activity of the 4-position alkyl-protons in the pyrazolin ring, as shown in Fig. 6. The keto form, which is usually dominant in organic solvents, has higher activity for HAT than the enol form. In aqueous media, on the analogy in alcoholic solvents, both keto and enol species might coexist, and in the high pH region (>7.0), edaravone can release a proton to form a mono-anion. The mono-anion form has higher activity for radical-scavenging than the neutral keto or enol form by means of the electron transfer (Fig. 6). Subsequently, the keto–enol tautomerism and acid–base dissociation equilibrium of edaravone are considered to be important for understanding the antioxidant action in living bodies because the contribution and the activity of the keto, enol, and anion forms should be different depending on the circumstances. The superior pharmacological activities of edaravone in vivo is due to its antioxidant activity, and relate to the interesting chemical property of edaravone that allows it to easily come and go between blood and tissues with switching its forms, i.e., keto, enol, and anion forms. It may be important that the pK_a value of edaravone equals 7.0 and is nearby the physiological pH of 7.4. Edaravone has the possibility to act as a good antioxidant both in water-rich regions, such as body fluids, and in hydrophobic regions, such as membranes inside of living bodies.

Conclusion

A kinetic study was performed for edaravone in order to clarify the mechanism of free-radical-scavenging and vitamin E-regenerating actions. The second-order rate constants for the radical-scavenging reaction of edaravone were measured in several organic solvents and in a water/methanol mixed solvent at various pH. The keto–enol tautomerism and the acid–base dissociation equilibrium of edaravone produce keto, enol, and anion forms in solutions, and their contributions and activities are varied depending on the properties of the solutions. From the results of NMR and kinetic studies, it has been clarified that the keto–enol tautomerism of edaravone actually exists, and the keto form has larger radical-scavenging activity than the enol form. Furthermore, the pH dependence of the rate constants suggests that the anion form produced by the acid–base dissociation equilibrium of edaravone has the highest radical-scavenging activity in the keto, enol, and anion forms.

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