Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one, Fig. 1) has been used as a free radical scavenging drug for the treatment of acute ischemic stroke in Japan since 2001. Edaravone is given to patients intravenously; therefore, it is distributed in the form of an aqueous solution. However, aqueous solutions of edaravone are very unstable because it is present as edaravone anion, which is capable of transferring an electron to free radicals including oxygen, and becomes edaravone radical. We observed the formation of hydrogen peroxide and edaravone trimer when aqueous edaravone solution was kept at 60°C for 4 weeks. We proposed the mechanism of edaravone trimer formation from edaravone radicals. Lowering the pH and deoxygenation can effectively increase the stability of aqueous edaravone solution, since the former reduces edaravone anion concentration and the latter inhibits edaravone radical formation. Addition of sodium bisulfite partially stabilized aqueous edaravone solutions and partially inhibited the formation of edaravone trimer. Formation of bisulfite adduct was suggested by 13C NMR and HPLC studies. Therefore, the stabilizing effect of sodium bisulfite is ascribed to the formation of a bisulfite adduct of edaravone and, consequently, reduction in the concentration of edaravone anion.

Key Words: edaravone, sodium bisulfite, bisulfite adduct, edaravone trimer, hydrogen peroxide

Stabilizers of edaravone aqueous solution and their action mechanisms. 1. Sodium bisulfite

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Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) has been used as a free radical scavenging drug for the treatment of acute ischemic stroke in Japan since 2001. Edaravone is given to patients intravenously; therefore, it is distributed in the form of an aqueous solution. However, aqueous solutions of edaravone are very unstable because it is present as edaravone anion, which is capable of transferring an electron to free radicals including oxygen, and becomes edaravone radical. We observed the formation of hydrogen peroxide and edaravone trimer when aqueous edaravone solution was kept at 60°C for 4 weeks. We proposed the mechanism of edaravone trimer formation from edaravone radicals. Lowering the pH and deoxygenation can effectively increase the stability of aqueous edaravone solution, since the former reduces edaravone anion concentration and the latter inhibits edaravone radical formation. Addition of sodium bisulfite partially stabilized aqueous edaravone solutions and partially inhibited the formation of edaravone trimer. Formation of bisulfite adduct was suggested by 13C NMR and HPLC studies. Therefore, the stabilizing effect of sodium bisulfite is ascribed to the formation of a bisulfite adduct of edaravone and, consequently, reduction in the concentration of edaravone anion.

Materials and Methods

Chemicals. Edaravone, sodium bisulfite, and other chemicals were of the highest grade commercially available. Water was purified with the Milli-Q Advantage system (Merck Millipore, Tokyo, Japan).

Stability of edaravone in aqueous solution. Edaravone (30 mg) was dissolved in 20 ml of water. If necessary, 1 N aqueous NaOH was added, and then the final pH was adjusted to 5–6 by adding 1 N aqueous HCl. The resulting 8.61 mM edaravone aqueous solution was mixed with 0 or 20 mg NaHSO3 (9.61 mM). Also, edaravone (30 mg) was dissolved in 2 ml of dimethylsulfoxide (DMSO) and mixed with 18 ml of water containing 0 or 20 mg NaHSO3. The above 4 solutions were placed in screw capped 50 ml vials and kept at 60°C for 4 weeks. To eliminate the effect of room light, the vials were covered by aluminum foil. Every week, pictures of the vials were taken (Fig. 2) and edaravone concentrations were measured by HPLC without removing precipitate if formed.

HPLC analysis. Edaravone was quantified by HPLC separation on a CAPCELL PAK ADME column (5 μm, 4.6 × 250 mm, Shiseido, Tokyo, Japan) using methanol/40 mM aqueous NaHPO4 (60/40 by volume) as the mobile phase (0.5 ml/min) with detection at 295 nm. Edaravone anion and sulfite adduct were measured by HPLC separation on an aminopropylsilyl column (LCNH4, 5 μm, 4.6 × 250 mm, Supelco Japan, Tokyo) using methanol/acetonitrile/40 mM aqueous NaHPO4 (45/50/5 by volume) as the mobile phase (1 ml/min) with detection at 240 nm.

LC/time-of-flight mass spectrometry (TOFMS) analysis. The precipitate was dissolved in methanol and analyzed by a reverse phase HPLC system combined with TOFMS (JMS-T100LC, JEOL Ltd., Tokyo, Japan). The mobile phase was 60% methanol containing formic acid (2.6 mM) and was delivered at a rate of 1.0 ml/min. An octadecylsilyl column (Wako, Osaka, Japan; 5 μm, 4.6 mm × 250 mm) was used for separation. Elution of metabolites was monitored by absorption at 210 nm using a UV
detector tandemly connected to TOFMS. Then, 1/4 of the eluent was induced into TOFMS. Negative ionization was performed at an ionization potential of $-2,000 \text{ V}$. The optimized applied voltages to the ring lens, outer orifice, inner orifice, and ion guide were $-5 \text{ V}$, $-10 \text{ V}$, $-5 \text{ V}$ and $-500 \text{ V}$, respectively. To obtain accurate $m/z$ values, trifluoroacetic acid was used as an internal standard for $m/z$ calibration.

**NMR analysis.** $^1\text{H}$ and $^{13}\text{C}$ NMR spectra were recorded on a Bruker Avance 600 spectrometer with BBO 600 MHz S3 5 mm probe (Bruker Biospin, Rheinstetten, Germany). All measurements were carried out at 25°C, and relaxation times were set at 1 s for $^1\text{H}$ and 2 s for $^{13}\text{C}$.

**Detection of hydrogen peroxide.** Hydrogen peroxide was measured using a specific HPLC method with isoluminol detection, as described previously. $^5$ The above LCNH$_2$ column was employed and methanol/40 mM aqueous NaHPO$_4$ (90/10 by volume) was used as the mobile phase (1 ml/min).

**Results and Discussion**

**Precipitation during storage.** Figure 2A shows the formation of a precipitate during the storage of 8.61 mM edaravone in water at 60°C for 4 weeks. More than 20% of edaravone decomposed during storage (Fig. 3A). Addition of 9.61 mM sodium bisulfite partially prevented the precipitate formation (Fig. 2A) and the degradation of edaravone (Fig. 3A) as compared with sodium bisulfite-free conditions. Addition of 10% DMSO did not affect the above results (Figs. 2B and 3B).

**Formation of edaravone trimer.** Next, we isolated the precipitate and subjected it to LC/TOFMS analysis. Figure 4A
showed the reversed-phase HPLC chromatogram of the precipitate monitored at 210 nm, indicating the precipitate contained two compounds. The $m/z$ of compound A with the shorter retention time was determined to be 173.10, which is identical with that of edaravone (173.10). Moreover, authentic edaravone gave identical retention time with compound A. Therefore, we concluded that compound A is edaravone. The $m/z$ of compound B with the longer retention time was determined to be 517.20 (Fig. 4B) and was identified as edaravone trimer [4,4-bis-(3-methyl-5-oxo-1-phenyl-2-pyrazolin-4-yl)-3-methyl-1-phenyl-2-pyrazolin-5-one, monoisotopic mass-1 = 344.19]. The fragment of 344.19 was assigned as edaravone dimer [4,4'-bis(3-methyl-1-phenyl-2-pyrazolin-5-one) anion radical (monoisotopic mass-1 = 173.10)]. Moreover, Heteronuclear Multiple Bond Coherence (HMBC) measurement revealed carbon 3 existed at 92 ppm as methine (3s). Since the peak height of carbon 1 was affected by the condition of carbon 3. Therefore, the chemical shift of carbon 4 reappeared, but at a different position, as shown in Fig. 7C. These results indicate that edaravone has at least two different forms in water, most likely the enol and edaravone anion forms in addition to the keto form. Interestingly, the chemical shift of carbon 1 was affected by the condition of carbon 3. Therefore, we named carbon 1 with methylene carbon 3 as 1(3d) and carbon 1 with methine carbon 3 as 1(3s). Since the peak height of carbon 1(3s) was higher than that of carbon 1(3d), the enol and edaravone anion forms were more predominant than the keto form. This result is acceptable because the solvent consisting of 10% DMSO-d$_6$ in heavy water (D$_2$O), the spectrum was drastically changed, as shown in Fig. 7B. The chemical shifts of carbons 3 and 4 disappeared, the chemical shift of carbons 1, 6, 7 and 8 were moved and split, and the chemical shift of carbon 2 was also changed. These results indicate that edaravone has at least two forms in water, most likely the enol and edaravone anion forms in addition to the keto form. Interestingly, the chemical shift of carbon 1 was affected by the condition of carbon 3. Therefore, we named carbon 1 with methylene carbon 3 as 1(3d) and carbon 1 with methine carbon 3 as 1(3s). Since the peak height of carbon 1(3s) was higher than that of carbon 1(3d), the enol and edaravone anion forms were more predominant than the keto form. This result is acceptable because the solvent consisting of 10% DMSO-d$_6$ in D$_2$O is protic and protic solvents stabilize the enol form of edaravone, and thus the enol form liberates proton and edaravone anion. Moreover, Heteronuclear Multiple Bond Coherence (HMBC) measurement revealed carbon 3 existed at 92 ppm as methine (3s) rather than methylene (3d) at 42 ppm. This result is consistent with the above notion that the enol and edaravone anion forms were predominant in water.

**Formation mechanism of edaravone trimer.** We propose the following mechanism of edaravone trimer formation from edaravone radical 2 (Fig. 6). The combination of two molecules of edaravone radical 2 gives edaravone dimer 1, which is then converted to the much more stable conjugated edaravone dimer 2 by enolization. Dimer 2 is easily converted to dimer radical 1, since dimer radical 1 is very stable due to conjugation with 4 double bonds. Dimer radical 1 is rearranged to dimer radical 2 and it gives edaravone trimer by combination with edaravone radical 2. It is noteworthy that the yield of edaravone dimer was very small compared to that of edaravone trimer. In fact, there were no significant peaks between edaravone and edaravone trimer (Fig. 4A). This result can be ascribed to the fact that edaravone dimer is much more reactive than edaravone.

**13C NMR spectrum of edaravone.** The keto form of edaravone is believed to be the most stable form in non-protic solvents. In fact, $^{13}$C NMR spectrum of edaravone in CDCl$_3$ shows eight single peaks, which are assigned as shown in Fig. 7A, and support the keto form of edaravone. When the solvent was changed to 10% DMSO-d$_6$, in heavy water (D$_2$O), the spectrum was drastically changed, as shown in Fig. 7B. The chemical shifts of carbons 3 and 4 disappeared, the chemical shift of carbons 1, 6, 7 and 8 were moved and split, and the chemical shift of carbon 2 was also changed. These results indicate that edaravone has at least two different forms in water, most likely the enol and edaravone anion forms in addition to the keto form. Interestingly, the chemical shift of carbon 1 was affected by the condition of carbon 3. Therefore, we named carbon 1 with methylene carbon 3 as 1(3d) and carbon 1 with methine carbon 3 as 1(3s). Since the peak height of carbon 1(3s) was higher than that of carbon 1(3d), the enol and edaravone anion forms were more predominant than the keto form. This result is acceptable because the solvent consisting of 10% DMSO-d$_6$ in D$_2$O is protic and protic solvents stabilize the enol form of edaravone, and thus the enol form liberates proton and edaravone anion. Moreover, Heteronuclear Multiple Bond Coherence (HMBC) measurement revealed carbon 3 existed at 92 ppm as methine (3s) rather than methylene (3d) at 42 ppm. This result is consistent with the above notion that the enol and edaravone anion forms were predominant in water.

**Addition of sodium bisulfite.** Sodium bisulfite reacts with carbonyl to give the sulfite adduct, as shown in Fig. 7. Since the keto and the amine forms of edaravone have carbonyl groups, two bisulfite adducts are possible. The addition of sodium bisulfite did not change the chemical shifts of edaravone carbons, except that the chemical shift of carbon 4 reappeared, but at a different position, as shown in Fig. 7C. These results indicate that the bisulfite adducts liberate carbonyl to some extent and the carbonyl should be different from the carbonyl located in the keto form of edaravone.
Fig. 6. Formation of edaravone trimer from edaravone radical 2 through edaravone dimer.

Fig. 7. $^{13}$C NMR spectra of edaravone in CDCl$_3$ (A), in DMSO-d$_6$/D$_2$O = 10/90 (v/v) (B), and in the presence of NaHSO$_3$ in DMSO-d$_6$/D$_2$O = 10/90 (v/v) (C).
Edaravone aqueous solution is unstable because of the presence of edaravone anion, which is capable of donating an electron to free radicals including oxygen and produces edaravone radical. We elucidated the mechanism of edaravone trimer formation from edaravone radicals. The addition of sodium bisulfite partially stabilized the aqueous edaravone solution and partially inhibited the formation of edaravone trimer. The formation of the bisulfite adduct was suggested by $^{13}$C NMR and HPLC studies, and this consequently reduces the concentration of edaravone anion.

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Abbreviations

ALS amyotrophic lateral sclerosis
DMSO dimethylsulfoxide
LCNH$_2$ aminopropylsilyl column
TOFMS time-of-flight mass spectrometry

Conflict of Interest

We have not received any financial support or other benefits from commercial sources for the work reported in this manuscript. None of the authors have financial interests that could create a potential conflict of interest or the appearance of a conflict of interest with regard to this work.

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