[Chem. Pharm. Bull.]
35(7)3045—3048(1987)

Photo-Stabilization and Solubilization of an Aldose Reductase Inhibitor, (*E*)-3-Carboxymethyl-5-[(2*E*)-methyl-3-phenylpropenylidene]rhodanine (ONO-2235), by Human Serum Albumin

YUKIHISA KURONO,* AKIHIRO FURUKAWA, YOKO TAKESUE,
FENGLONG LI, and KEN IKEDA

*Faculty of Pharmaceutical Sciences, Nagoya City University,
3-1, Tanabe-dori, Mizuho-ku, Nagoya 467, Japan*

(Received December 9, 1986)

Photo-stabilization and solubilization of an aldose reductase inhibitor, (*E*)-3-carboxymethyl-5-[(2*E*)-methyl-3-phenylpropenylidene]rhodanine (ONO-2235), by human serum albumin (HSA) were investigated in aqueous solution. ONO-2235 dissolved in pH 7.4 phosphate buffer was photo-stabilized by a factor of about 150 in the presence of a 5-fold excess of HSA per mole of the drug. The solubilities of ONO-2235 in the buffer and distilled water were increased about 17-fold and 57-fold, respectively, by the presence of 6×10^{-4} M or more HSA. The binding parameters for the interaction between ONO-2235 and HSA were determined from equilibrium dialysis data. These results indicate the usefulness of HSA as a drug additive.

Keywords—(*E*)-3-carboxymethyl-5-[(2*E*)-methyl-3-phenylpropenylidene]rhodanine (ONO-2235); human serum albumin; solubility; solubilization; photo-stability; stabilization; drug additive; binding; binding parameter; equilibrium dialysis

Human serum albumin (HSA) is a major protein component of human blood, and strongly binds numerous endogenous and exogenous compounds.¹⁻³⁾ (*E*)-3-Carboxymethyl-5-[(2*E*)-methyl-3-phenylpropenylidene]rhodanine (ONO-2235) is an aldose reductase inhibitor and is expected to be used therapeutically in some diabetic complications.^{4,5)} However, ONO-2235 is photo-unstable and has low water solubility. As a part of our continuing studies on the utilization of HSA as a drug additive (e.g., as a solubilizer and stabilizer),⁶⁻⁸⁾ we examined its effect on the properties of ONO-2235. In this paper, we report the photo-stabilization and solubilization of ONO-2235 by HSA, and also the binding parameters for the interaction between ONO-2235 and HSA.

Experimental

Materials—HSA (Sigma Chemical Co., lot 35F-9432) was used after purification by the method of Chen.⁹⁾ The concentration of HSA was determined by use of its molar absorptivity ($\epsilon_{\text{HSA}} = 3.66 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) at 278 nm, assuming a molecular weight of 69000.¹⁰⁻¹²⁾ ONO-2235 (lot J-3006) was supplied by Ono Pharmaceutical Co., Ltd. and was used without further purification. All other chemicals were purchased and were of reagent grade.

Analysis of ONO-2235—Since ONO-2235 exhibits absorption maxima at 400 nm ($\epsilon_{\text{ONO-2235}} = 4.04 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) and 294 nm ($\epsilon'_{\text{ONO-2235}} = 1.13 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) and the photo-decomposition products do not have absorbance at 400 nm, the absorbance at 400 nm was used for the quantitative analysis of ONO-2235 in the absence of HSA. In the presence of HSA, the absorbance at 433 nm was employed because the spectra of HSA-bound ONO-2235 and unbound ONO-2235 show an isosbestic point at 433 nm ($\epsilon_{\text{mixture}} = 1.68 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$).

High-performance liquid chromatography (HPLC) was carried out for the separation of ONO-2235 and its photo-decomposition products. The apparatus (TRI ROTOR®, JASCO) was equipped with a Cosmosil C_{18} column (Nakarai Kagaku Inc., 5 μm , 4.6 i.d. $\times$ 150 mm) and a variable-wavelength spectrophotometric detector (UVDEC 100-II, JASCO), operated at 294 nm. The mobile phase was pH 7.4, 0.01 M phosphate buffer : acetonitrile (65 : 35, v/v)

and the flow rate was 1.0 ml/min. Peak area analyses were carried out with a C-R1B data processor (Shimadzu). The injection reproducibility was within $\pm 1\%$. Under these conditions the retention time, t_R , of ONO-2235 was about 7.5 min, and two other peaks due to the photo-decomposition products were observed at t_R 's of about 5.5 and 6.5 min. The chemical structures of the products are unknown at present. The peak area of t_R 7.5 min was used for the quantitative analysis of ONO-2235.

Photo-Reaction—The photo-reaction was carried out in an irradiation apparatus (Riko, Type UVL-100P). The buffered ONO-2235 solution in the presence and absence of HSA was placed in the reaction vessel (made from quartz), and was photo-irradiated with a 100 W mercury vapor lamp (Riko, Type UVL-100H). At appropriate time intervals, samples were withdrawn and were analyzed by the ultraviolet spectrum (UV) method or HPLC method described above. The reaction temperature was 21–27 °C.

Solubility—ONO-2235 (10 mg) was placed in vials containing 3 ml of $0\text{--}9.40 \times 10^{-4}$ M HSA solution prepared from the pH 7.4 buffer or distilled water. The vials were shaken for about 20 h in a thermostated room at 25 ± 1 °C. The samples were filtered with a Millipore filter (Millipore Corporation, $0.5 \mu\text{m}$) and diluted with the pH 7.4 buffer. The concentrations of ONO-2235 were determined by the UV method.

Equilibrium Dialysis—Visking® cellulose tubing (Viskase Corporation) was used after pretreatment (it was boiled for 30 min twice in distilled water and in phosphate buffer).^{12,13} The compartments for the dialysis contained 10 ml of 7.40×10^{-6} – 9.90×10^{-5} M HSA solution (inside) and 40 ml of 9.00×10^{-6} – 9.70×10^{-5} M ONO-2235 solution (outside). After 17–23 h under shaking in a room at 25 ± 1 °C, the concentration of ONO-2235 outside was determined by the UV method.

Results and Discussion

Photo-Stabilization

Figure 1 shows the first-order plot for the photo-decomposition of ONO-2235 in the presence and absence of HSA. All the plots are linear, and the pseudo first-order rate constant, k_{obs} , was determined according to Eq. 1.

$$\log(A_t/A_0) = -k_{\text{obs}} \cdot t/2.303$$

In Eq. 1, A_0 and A_t are the peak areas (or absorbances) at times 0 and t , respectively. The k_{obs} values in the absence of HSA, determined from the plots based on the UV method (●) and the HPLC method (○), were $2.45 \times 10^{-3} \text{ s}^{-1}$ (half life, $t_{1/2} = 2.83 \times 10^2 \text{ s}$) and $2.33 \times 10^{-3} \text{ s}^{-1}$ ($t_{1/2} = 2.97 \times 10^2 \text{ s}$), respectively. Both values are in good agreement with each other. In the presence of HSA the value of k_{obs} was $1.62 \times 10^{-5} \text{ s}^{-1}$ ($t_{1/2} = 4.28 \times 10^4 \text{ s} = 11.9 \text{ h}$), which is about 1/150 of that in the absence of HSA. It is apparent that the photo-stabilizing effect of HSA is very large.

Solubilization

Figure 2 shows the effect of HSA concentration on the solubility of ONO-2235. In both the buffer and distilled water, maximum solubility (about 3.80×10^{-3} M) was obtained at 6×10^{-4} M or more HSA concentration. In the absence of HSA the solubilities in the buffer and distilled water are about 2.25×10^{-4} M and 6.66×10^{-5} M, respectively. The differences in the values between the buffer and distilled water may result from the difference in the degree

Fig. 1. First-Order Plots for the Photo-Decomposition of ONO-2235 Irradiated with a UVL-100H Lamp at 21–27 °C in 0.067 M Phosphate Buffer, pH 7.4 ($\mu = 0.2$ with NaCl)

○, 2.00×10^{-5} M ONO-2235 (determined by HPLC method); ●, 2.50×10^{-5} M ONO-2235 (determined by UV method); ▲, 2.50×10^{-5} M ONO-2235 + 1.25×10^{-4} M HSA (determined by the UV method).

Fig. 2. Effects of HSA Concentration on the Solubility of ONO-2235 at 25 °C

●, 0.067 M phosphate buffer (pH 7.4); ○, distilled water.

Fig. 3. Scatchard Plot for Binding of ONO-2235 to HSA Using Equilibrium Dialysis Data at 25 °C

The solid curve was calculated by using the obtained parameters.

of dissociation of the carboxyl group in the ONO-2235 molecule. The ratios of the maximum solubility to the solubilities of ONO-2235 in the absence of HSA are about 17 and 57 in the buffer and distilled water, respectively. The solubilizing effect of HSA is large.

Binding Parameters for Interaction between ONO-2235 and HSA

Since the binding of ONO-2235 to HSA is considered to be one of the mechanisms of the photo-stabilization and solubilization of ONO-2235 by HSA, equilibrium dialysis was carried out for the determination of the binding parameters. Figure 3 shows the Scatchard plot¹²⁻¹⁵⁾ for the equilibrium dialysis data, where r represents moles of bound ONO-2235 per mole of HSA and C_f is the unbound (free) concentration of ONO-2235. The curvature of the plot indicates the presence of more than two classes of binding sites. When two classes of binding sites on HSA are assumed, the following equation¹²⁻¹⁵⁾ can be used for the analysis.

$$r = n_1 K_1 C_f / (1 + k_1 C_f) + n_2 K_2 C_f / (1 + K_2 C_f) \quad (2)$$

In Eq. 2, n and K represent the number of the binding sites and the association constant, respectively. Subscripts 1 and 2 indicate the high- and low-affinity binding sites, respectively. The binding parameters (n_i and K_i) were determined by applying non-linear least-squares analysis (MULTI¹⁶⁾) to Eq. 2. The obtained values are: $n_1 = 2.12$, $K_1 = 1.12 \times 10^6 \text{ M}^{-1}$, $n_2 = 8.38$ and $K_2 = 5.41 \times 10^4 \text{ M}^{-1}$. These values indicate that there is a strong interaction between ONO-2235 and HSA.

In conclusion, the binding of ONO-2235 to HSA leads to the photo-stabilization and solubilization of ONO-2235, indicating the usefulness of HSA as a new drug additive.

Acknowledgments The authors are grateful to Ono Pharmaceutical Co., Ltd. for the gift of ONO-2235. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

References and Notes

- 1) U. Kragh-Hanse, *Pharmacol. Rev.*, **33**, 17 (1981).
- 2) H. Terada, K. Hiramatsu, K. Ikeda, M. Otagiri, and S. Goto, "Serum Albumin," ed. by K. Aoki, T. Takagi, and H. Terada, Kodansha, Tokyo, 1984, pp. 81—160.
- 3) T. Peters, Jr., *Adv. Prot. Chem.*, **37**, 161 (1985).
- 4) H. Terashima, K. Hama, R. Yamamoto, H. Tsuboshima, R. Kikkawa, I. Hatanaka, and Y. Shigeta, *J. Pharmacol. Exp. Ther.*, **229**, 226 (1984).
- 5) T. Asano, S. Mochio, T. Kuwata, H. Oka, S. Hattori, M. Abe, and M. Terashima, *Tonyobyō*, **26**, 1105 (1983) [*Chem. Abstr.*, **100**, 132471x (1984)].
- 6) Y. Kuroono, N. Ohta, and K. Ikeda, *Chem. Pharm. Bull.*, **30**, 2635 (1982).
- 7) K. Ikeda, T. Koide, and K. Inaba, Jpn. Kokai Tokkyo Koho, Japan. Patent, 58216126 [*Chem. Abstr.*, **100**, 109119e (1984)].
- 8) N. Ohta, T. Yotsuyanagi, and K. Ikeda, *Chem. Pharm. Bull.*, **34**, 2585 (1986).
- 9) R. F. Chen, *J. Biol. Chem.*, **242**, 173 (1967).
- 10) G. E. Means and M. L. Bender, *Biochemistry*, **14**, 4989 (1975).
- 11) Y. Kuroono, K. Ichioka, and K. Ikeda, *J. Pharm. Sci.*, **72**, 432 (1983).
- 12) F. Li, Y. Kuroono, A. Furukawa, and K. Ikeda, *Yakugaku Zasshi*, **106**, 427 (1986).
- 13) Y. Kuroono, N. Ohta, T. Yotsuyanagi, and K. Ikeda, *Chem. Pharm. Bull.*, **29**, 2345 (1981).
- 14) G. Scatchard, J. C. Coleman, and A. L. Shen, *J. Am. Chem. Soc.*, **79**, 12 (1957).
- 15) I. M. Klotz and D. L. Humston, *Biochemistry*, **10**, 3065 (1971).
- 16) K. Yamaoka, Y. Tanigawara, T. Nakagawa, and T. Uno, *J. Pharmacobio-Dyn.*, **4**, 879 (1981).