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pH-Dependent Degradation and Stabilization of Meclofenoxate Hydrochloride by Human Serum Albumin

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The hydrolysis rate of meclofenoxate hydrochloride (MFX) was investigated in the absence and in the presence of human serum albumin (HSA). Compared with the degradation of the drug (pK_a 8.2) in buffer solutions, the degradation rate in HSA solutions (pH 4.0—10.7) was accelerated in the acidic region below pH 7.0 and was retarded in the basic region above pH 7.0.

Based on a Michaelis–Menten type kinetic scheme involving the formation of HSA–drug complexes for both protonated and neutral forms of MFX, the effects of the protein on the degradation rate were analyzed and the hydrolysis rate constants and the binding constants of HSA–drug complexes were obtained.

It was found that the neutral form was considerably stabilized by the association with HSA whereas the degradation of the protonated form was accelerated, and the neutral form–HSA complex was extremely stable compared with the protonated form–HSA complex. These results suggest that the neutral and protonated forms are bound to HSA molecules in different modes, the former being less accessible than the latter to hydroxide ion attack.

Keywords——meclofenoxate hydrochloride; human serum albumin: drug stability; kinetics; protein binding

Meclofenoxate hydrochloride (MFX), a cerebral stimulant, penetrates into the central nervous system in the form of the ester, then it is hydrolyzed to *p*-chlorophenoxyacetic acid (CPA) and *β*-dimethylaminoethanol (DMAE), an antidepressant. The brain level of DMAE was found to be several-fold higher in treatment with MFX than with DMAE.[1] Accordingly, the degradation behavior of MFX in the circulation is closely related to its biological activity.

It is known that albumin has a non-specific esterase-like activity toward some esters and amides such as *p*-nitrophenyl acetate,[2,3] 5-nitrosalicyl esters,[4] and *N-trans*-cinnamoylimidazoles.[5] On the other hand, some drugs such as thromboxane A_2,[6] PGI_2[7] and melphalan[8] were found to be stabilized in the presence of albumin. It has been recently suggested that the hydrolysis of MFX is retarded by serum protein at pH 7.4.[9] In view of the utilization of albumin as a drug additive, we studied the effect of human serum albumin (HSA) on the hydrolysis of MFX. In aqueous solution (pH 7.4), $t_{1/2}=16$ min at 25 °C, the degradation of the drug was enhanced in the acidic region but decreased in the basic region. The contribution of HSA to the stability of MFX in blood is discussed.

Experimental

Materials——MFX was a gift from Dainippon Pharmaceutical Co., Osaka. HSA (Fraction V, lot #64F-9309) was purchased from Sigma Chemical Co., and used after purification by the method of Chen.[10] The concentration of HSA was determined from the absorption at 278 nm ($E^{0.1\%}_{1\,cm}=0.531$)[2] and the molecular weight was assumed to be 69000. Acetonitrile was of high-performance liquid chromatography (HPLC) quality. All other chemicals used were of reagent grade.

Kinetic Procedure——All kinetic studies were carried out at 25±0.2 °C. The reactions were initiated by mixing

MFX stock solution (pH 3.0) with buffer or buffered albumin solution preincubated at 25±0.2 °C. The final concentration of HSA was more than 6 times that of MFX (1.5×10^{-4} M). At suitable intervals, aliquots were withdrawn, diluted, deproteinized with acetonitrile, and centrifuged at $1100\times g$ for 10 min, then 50 μl of the clear supernatant was injected into the chromatograph. Michaelis buffer (pH 4.0—5.0), Sörensen buffer (pH 6.3—8.3) and Kolthoff buffer (pH 9.3—11.8) were used as reaction media. The ionic strength was adjusted to 0.2 with sodium chloride.

Pseudo-first-order rate constants were calculated from the slope of linear plots of the logarithm of residual MFX against time.

HPLC——Chromatography was performed with a system consisting of a TRI ROTOR pump (JASCO, Tokyo, Japan) equipped with a Unisil Q C_{18} column (Gasukuro Kogyo Inc., Tokyo, Japan, 5 μm, 4.6×250 mm) and a JASCO UVIDEC 100-II variable-wavelength spectrophotometric detector (JASCO) operated at 280 nm. The mobile phase consisted of acetonitrile–acetate buffer, pH 4.0 (35 : 65, v/v), containing 15 mM sodium 1-heptanesulfonate. The flow rate was 0.7 ml/min. Peak areas of MFX were analyzed with a C-R1B data processor (Shimadzu, Kyoto, Japan). The injection reproducibility was within ±1%. *p*-Methylbenzoic acid was used as an internal standard. MFX, CPA and *p*-methylbenzoic acid had elution times of 5.1, 3.4 and 7.2 min, respectively.

Results and Discussion

Hydrolysis of MFX in Aqueous Solution

The hydrolytic degradation of MFX is known to follow first-order kinetics, producing CPA and DMAE.[11] The degradation of MFX was examined in buffer solutions of pH 4.0—11.9 to evaluate the effect of HSA on the hydrolytic behavior of the drug, as described in the next section. The observed first-order rate constant was influenced by the buffer concentrations, as shown in Fig. 1, in both phosphate (pH 8.3) and borate (pH 10.3) buffers. By extrapolatings to the ordinate (buffer concentration assumed to be 0 M), the buffer-free rate constant, k_s, was obtained and is used as a basis for discussion hereinafter.

The pH dependence of k_s is shown in Fig. 2. In the pH region of 5.5—7.4, the pH profile for the hydrolysis of MFX was linear with a slope of about 1, which means that the contribution of specific base catalysis is predominant in this region. This pH–rate profile could be accounted for by assuming base-catalyzed decomposition of both protonated and neutral forms of MFX, the pK_a value being 8.3,[11] as illustrated in Chart 1, and the following rate expression may be derived

$$k_s=\frac{k_h[OH^-]}{1+K_a/[H^+]}+\frac{k_0[OH^-]}{1+[H^+]/K_a} \quad (1)$$

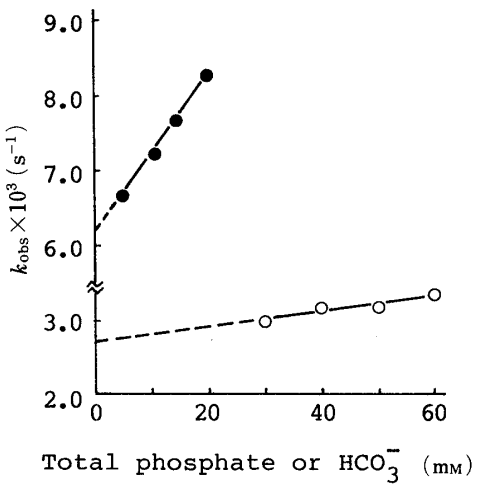

Fig. 1. Effect of Buffer Concentration on the Observed Rate Constant

○, pH 8.3 (Na_2HPO_4–KH_2PO_4); ●, pH 10.3 (Na_2CO_3–$Na_2B_4O_7$). Ionic strength, 0.2 and 25 °C.

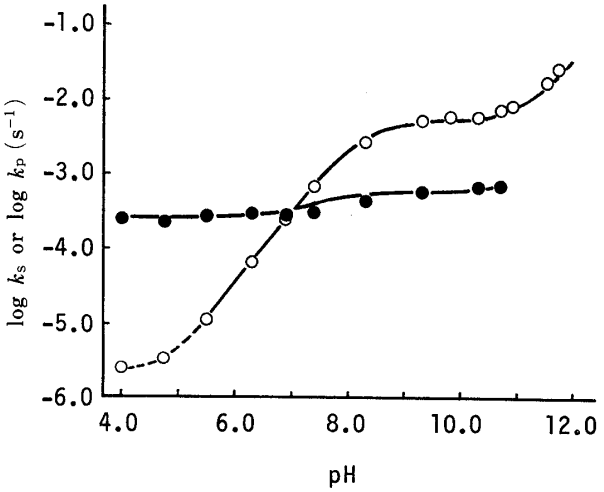

Fig. 2. The pH–Rate (k_s or k_p) Profiles for the Hydrolysis of MFX at 25 °C

HSA: ○, 0 M; ●, 3.0×10^{-3} M.

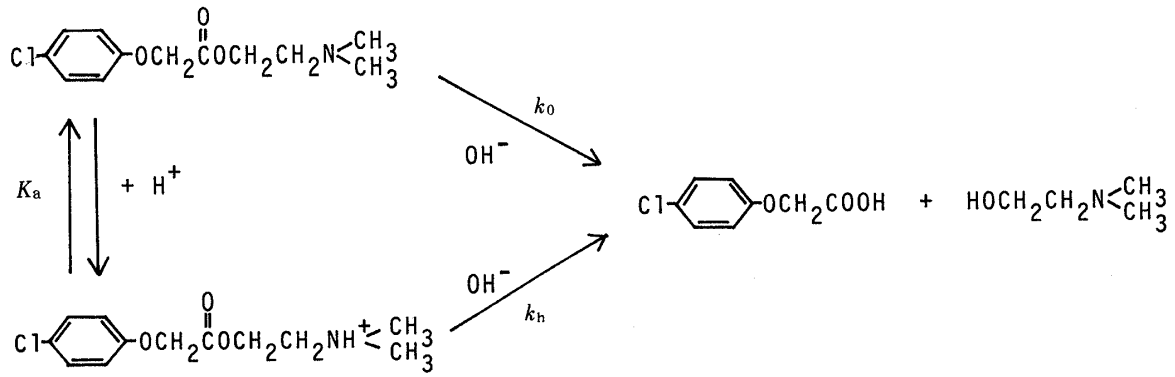

Chart 1

TABLE I. Association Constants and Rate Constants for the Hydrolysis of MFX in Aqueous Solution and in HSA Solution at 25 °C

K_a	6.03×10^{-9} M (pK_a 8.2)
k_h	3.34×10^{3} $M^{-1} s^{-1}$
k_0	3.69 $M^{-1} s^{-1}$
K_{c+}	3.33×10^{2} M^{-1}
k_{c+}	5.68×10^{-4} s^{-1}
K_c	2.73×10^{3} M^{-1}
k_c	$\simeq 0$ s^{-1}

where $[H^+]$ and $[OH^-]$ are hydrogen ion and hydroxide ion concentrations, respectively, k_0 and k_h are the second-order rate constants of free and protonated forms of MFX for specific base-catalysis, respectively, K_a is the protolytic dissociation constant, and $K_w = 1.01 \times 10^{-14}$ at 25 °C.[12] The values of the rate constants and K_a derived kinetically from Fig. 2 and Eq. 1 are listed in Table I.

The kinetically derived pK_a agrees satisfactorily with pK_a 8.3 (ionic strength, 0.6 at 29.1 °C) determined by Yamana *et al.*[11] The solid line in the absence of HSA drawn in Fig. 2 represents the calculated profile based on Eq. 1 and the values in Table I.

Effect of HSA on the Hydrolysis of MFX

The hydrolysis of MFX was investigated at pH 5.5 and 10.3 in the presence of various concentrations of HSA at 25 °C. Under the given reaction conditions, strict first-order kinetics was observed up to at least 80% degradation of the drug, from which the observed first-order rate constant was determined (Fig. 3). The buffer-free rate constant (k_p) was also obtained in the presence of HSA. At pH 5.5 HSA facilitated the hydrolysis of MFX. The rate increased with increasing HSA concentration in a nonlinear fashion to reach a maximum value (Fig. 4). However, at pH 10.3 HSA stabilized MFX, reaching a minimum value with increasing HSA concentration (Fig. 5). The dependency of k_p on pH in the range of 4.0—10.7 was determined at the concentrations of MFX and HSA of 1.5×10^{-4} M and 3.0×10^{-3} M, respectively, as shown in Fig. 2. HSA showed opposite effects on the stability of MFX in relation to that at pH 7.0. In view of the pK_a value (8.2) of MFX, the rate enhancement by HSA involved the protonated form as the predominant species, while the rate was retarded at pH values of more than 7.0, where the neutral form is increasingly prominent. It should be noted that k_p was little dependent on pH in the range of 4.0—10.7.

On the basis of the assumption that a 1 : 1 complex is formed between HSA and MFX (both protonated and free form), the observed degradation and stabilization of MFX by HSA may be accommodated by the reaction mechanism illustrated in Chart 2, where K_{c+} and K_c

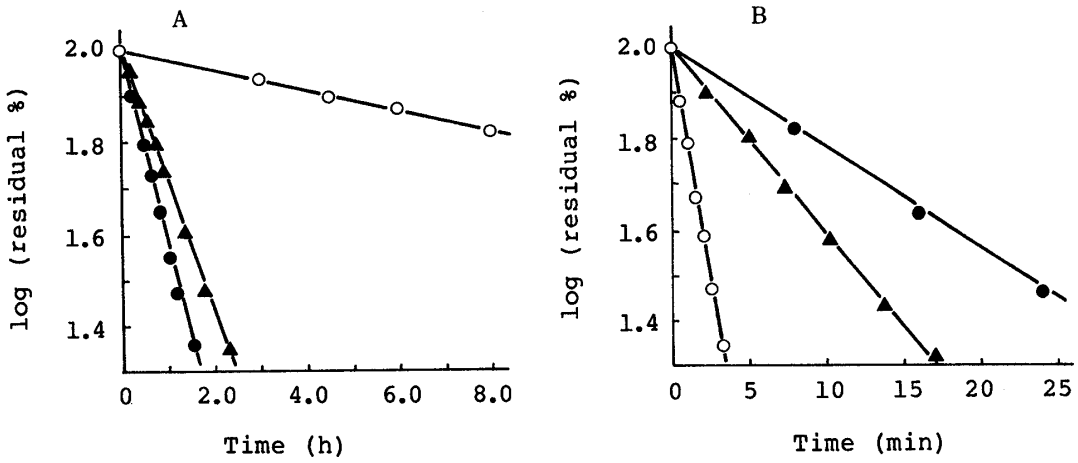

Fig. 3. First-Order Plots for the Hydrolysis of MFX in the Absence and Presence of HSA at 25 °C

A; pH 5.5 HSA: ○, 0 M; ▲, 1.4×10^{-3} M; ●, 3.1×10^{-3} M. MFX, 1.5×10^{-4} M; 50 mM acetate buffer.
B; pH 10.3 HSA: ○, 0 M; ▲, 1.5×10^{-3} M; ●, 3.0×10^{-3} M. MFX, 1.5×10^{-4} M; 37.5 mM borate buffer.

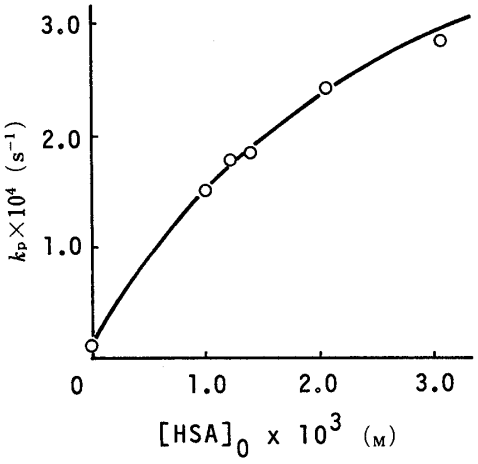

Fig. 4. Effect of HSA Concentration on the Hydrolysis of MFX at pH 5.5

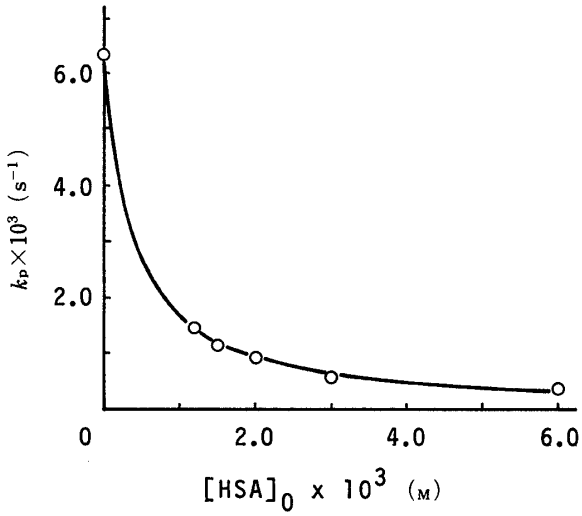

Fig. 5. Effect of HSA Concentration on the Hydrolysis of MFX at pH 10.3

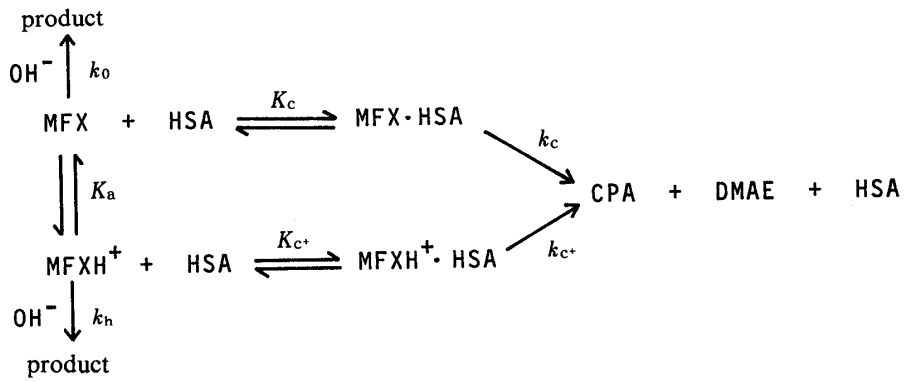

Chart 2

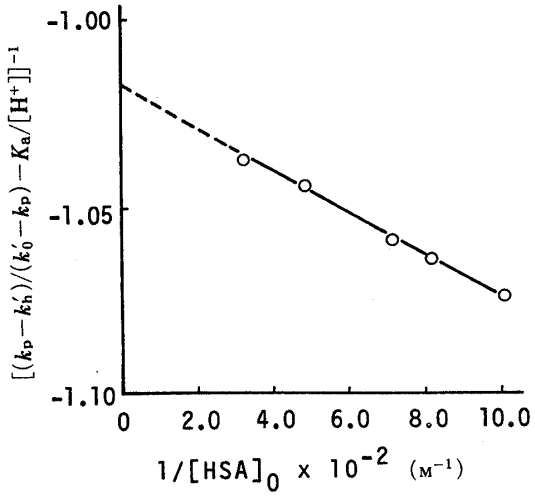

Fig. 6. Double-Reciprocal Plots of the Data in Fig. 4 according to Eq. 4 at pH 5.5

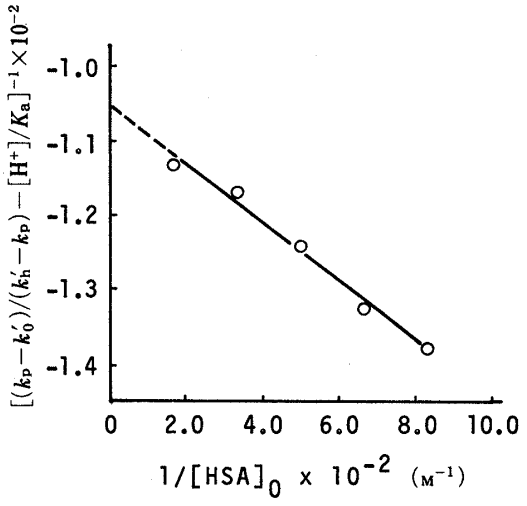

Fig. 7. Double-Reciprocal Plots of the Data in Fig. 5 according to Eq. 6 at pH 10.3

are the binding constants for the complexes formed between protonated and neutral MFX and HSA, and k_{c+} and k_c are the pseudo-first-order rate constants for the hydrolysis of respective complexes. According to this scheme, the pseudo-first-order rate constant, k_p, is given by Eq. 2.

$$k_p=\frac{k_0'+k_h'[H^+]/K_a+k_cK_c[HSA]_0+k_{c+}K_{c+}[HSA]_0[H^+]/K_a}{1+[H^+]/K_a+K_c[HSA]_0+K_{c+}[HSA]_0[H^+]/K_a} \tag{2}$$

where k_0' and k_h' represent $k_0[OH^-]$ and $k_h[OH^-]$, respectively. In the presence of a large excess of HSA compared to MFX, the HSA concentration could be replaced by the initial concentration. To determine the kinetic parameters, further mathematical manipulation of Eq. 2 was done under the following assumptions: in the region of $pH \ll pK_a$, where the free form of the drug should be negligible, Eq. 2 simplifies to

$$k_p=\frac{k_0'+k_h'[H^+]/K_a+k_{c+}K_{c+}[HSA]_0[H^+]/K_a}{1+[H^+]/K_a+K_{c+}[HSA]_0[H^+]/K_a} \tag{3}$$

and the reciprocal form is derived as

$$\frac{1}{(k_p-k_h')/(k_0'-k_p)-K_a/[H^+]}=\frac{(k_h'-k_0')[H^+]/K_a}{\{k_0'-k_{c+}+(k_h'-k_{c+})[H^+]/K_a\}K_{c+}}\cdot\frac{1}{[HSA]_0}+\frac{(k_{c+}-k_0')[H^+]/K_a}{k_0'-k_{c+}+(k_h'-k_{c+})[H^+]/K_a} \tag{4}$$

If $pH \gg pK_a$, where the protonated form is not involved in the reaction, the corresponding equation is

$$k_p=\frac{k_0'+k_h'[H^+]/K_a+k_cK_c[HSA]_0}{1+[H^+]/K_a+K_c[HSA]_0} \tag{5}$$

and the reciprocal equation is

$$\frac{1}{(k_p-k_0')/(k_h'-k_p)-[H^+]/K_a}=\frac{k_h'-k_0'}{\{k_c-k_0'-(k_h'-k_c)[H^+]/K_a\}K_c}\cdot\frac{1}{[HSA]_0}+\frac{k_h'-k_c}{k_c-k_0'-(k_h'-k_c)[H^+]/K_a} \tag{6}$$

A plot of the left-hand term of Eq. 4 or 6 against $1/[HSA]_0$ should give a straight line. Figures 6 and 7 show plots of the data. From the value of the intercept, k_{c+} or k_c was estimated, and from the slope K_{c+} or K_c was estimated. The parameters are listed in Table I. The solid curves in Figs. 4 and 5 were calculated by means of Eqs. 3 and 5 using the parameters, and show good agreement with the experimental data. Furthermore, these parameters were introduced into Eq. 2 and k_p values were calculated over the pH range examined in the presence of 3.0×10^{-3} M HSA. The calculated solid line is in good accordance with the experimental values (Fig. 2), indicating that the model shown in Chart 2 for the hydrolytic behavior of MFX in the presence of HSA is reasonable.

The N–B transition of the HSA molecule occurs between pH 6 and 9.[13,14] A two-state conformational model was applied for the binding of warfarin to HSA,[15] in which pH-dependent binding constants for the N and B forms were assumed. In a similar treatment, the K_c value of MFX obtained at pH 10.3 could represent the binding constant to the B form of HSA. If the binding constant was calculated as a function of the fraction of either N or B form with different affinities, the calculated pH profile of k_p did not give a good fit to the experimental values, especially in the pH region above 7.5 where the fraction of B form increases. Accordingly, there seems to be little difference between the binding constants of MFX to the N and B forms of HSA.

On the basis of the hydrolysis rate constants of the MFX species, the free form was considerably stabilized by the association with HSA whereas the degradation of the protonated form was accelerated. These results suggest that the ester linkage of the neutral form was buried in or shielded by the HSA molecule. On the other hand, the protonated form may be associated with the protein in a different binding mode where the species is more susceptible to hydroxide ion attack, even if the binding site of both species is the same. Therefore, the observed stabilization of MFX by HSA in the basic region arises from the negligible hydrolysis rate of the neutral form–HSA complex and larger binding constant as compared with that of the protonated form–HSA complex.

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