Epimerization and Racemization of Some Chiral Drugs in the Presence of Human Serum Albumin

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Epimerization and racemization of carbenicillin, ethiazide, etoposide and oxazepam acetate were examined kinetically in the presence of human serum albumin (HSA). The concentration of both optical isomers of each drug was determined by stereospecific high-performance liquid chromatography. The apparent rate constants of epimerization or racemization and hydrolysis were estimated from the concentration–time data. HSA retarded the racemization of ethiazide and the epimerization of etoposide. The binding of the drugs to HSA may inhibit the attack of hydroxy ion and/or water molecule and thus retard the epimerization and the racemization. HSA accelerated the epimerization of carbenicillin, which is charged at the pH studied. Ion–ion and ion–dipole interactions between carbenicillin and HSA activate the carbenicillin molecule favorable for the attack of hydroxy ion and/or water molecule. The hydrolysis rates of ethiazide, carbenicillin and oxazepam acetate were increased by the addition of HSA. The hydrolysis rate of *d*-oxazepam acetate enantiomer bound to HSA was twice that of the *l*-enantiomer, which suggests that the esterase-like activity of HSA is enantioselective. Differences in the binding affinities of the drug's enantiomers to HSA may account for the selectivity.

Keywords epimerization; racemization; hydrolysis; kinetics; carbenicillin; ethiazide; etoposide; picroetoposide; oxazepam acetate; HSA

For many chiral drugs, the pharmacological effect and pharmacokinetics differ between the two isomers.[1,2] It has been reported that etoposide has more biological activity than its epimeric isomer, picroetoposide.[3] The pharmacological effects of structurally related compounds of carbenicillin, ethiazide and oxazepam acetate also differ between the two isomers of each compound.[4-6] It is thus of great interest to study the kinetics of epimerization or racemization of such drugs and important to elucidate the factors affecting the epimerization and racemization of chiral drugs.

Epimerization and racemization of many chiral drugs have been studied, including pilocarpine hydrochloride,[7] etoposide,[8] tetracycline,[9] moxalactam,[10] cefsulodin,[11] oxazepam,[12] adrenaline and hyoscyamine.[13] These studies have indicated that acid and base catalysis are important factors affecting epimerization and racemization of chiral drugs.

Human serum albumin (HSA), which is the most abundant protein in blood plasma, has an esterase-like activity toward many compounds, such as *p*-nitrophenyl acetate,[14,15] aspirin derivatives[16] and *N-trans*-cinnamoylimidazoles.[17] HSA affects the stability of many drugs, *e.g.* meclofenoxate hydrochloride[18,19] and gabexate mesilate,[20] and thus may possibly affect the epimerization and racemization of chiral drugs. Few papers, however, have reported the effects of HSA on the epimerization or racemization of drugs.[21]

In the present study, epimerization or racemization of carbenicillin, ethiazide, etoposide and oxazepam acetate were examined kinetically in the presence of HSA. Apparent rate constants of epimerization or racemization and hydrolysis of the drugs were estimated from the time–concentration profiles for both optical isomers of the drugs, which were determined by stereospecific high-performance liquid chromatography (HPLC). After presentation of these results, we discuss the effects of HSA on the epimerization and racemization, and the mechanism of the interaction between HSA and the drugs.

Materials and Methods

Materials Carbenicillin and HSA (fraction V, essentially fatty acid-free) were obtained from Wako Pure Chemical Industries (Osaka, Japan) and Sigma Chemical Co. (St. Louis, U.S.A.), respectively. The molecular weight of HSA was assumed to be 69000.[15] Ethiazide, etoposide, picroetoposide and oxazepam were gifts from manufacturers (ethiazide, Tokyo Tanabe Pharmaceutical Co., Tokyo, Japan; etoposide and pic-

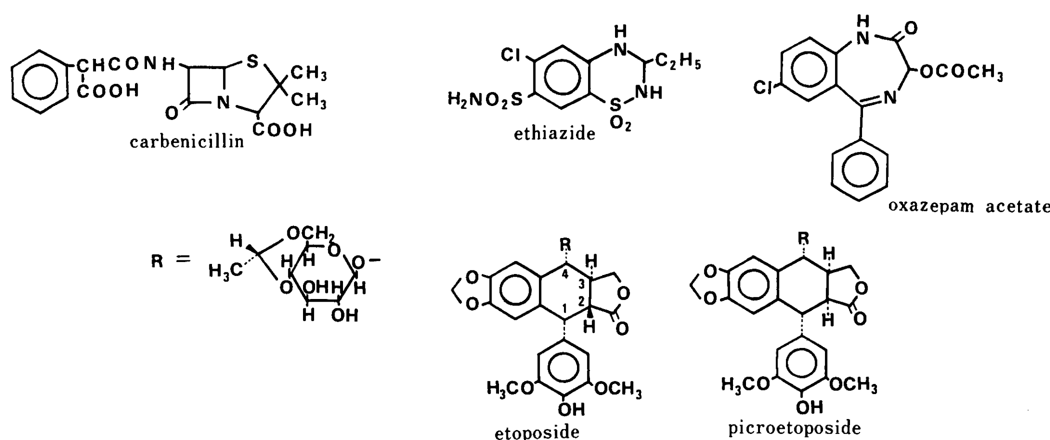

Fig. 1. Structures of Drugs Studied

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TABLE I. Chromatographic Conditions

Drug	Stationary phase	Column temp. (°C)	Mobile phase solvent	Detection
Carbenicillin	TSKgel ODS-80TM[a] 4.6 × 150 mm	35	0.05 M Phosphate (pH 7.0)–MeOH (4 : 1)	220 nm
Etoposide	TSKgel ODS-80TM 4.6 × 150 mm	35	0.05 M Acetate (pH 4.0)–acetonitrile (2 : 1)	290 nm
Ethiazide	Sumipax OA-2000[b] 4 × 250 mm	30	Hexane–CH_2Cl_2–MeOH–AcOH (5 : 4 : 1 : 0.1)	270 nm
Oxazepam acetate	Sumipax OA-2000 4 × 250 mm	30	Hexane–CH_2Cl_2–iso-PrOH–EtOH (5 : 5 : 1 : 1)	254 nm

a) An octadecyl silane bonded phase (Tosoh, Tokyo, Japan). *b*) *N*-3,5-Dinitrobenzoyl-(*R*)-phenylglycine phase (Sumika Chemical Analysis Service Ltd., Osaka, Japan).

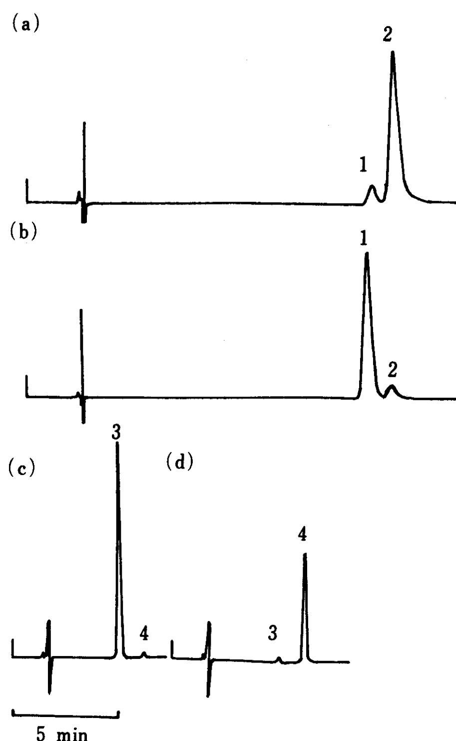


Fig. 2. Typical Chromatograms of Ethiazide and Carbenicillin Sample Obtained

(a) *d*-ethiazide fraction, (b) *l*-ethiazide fraction, (c) D-carbenicillin fraction, (d) L-carbenicillin fraction. 1, *l*-ethiazide; 2, *d*-ethiazide; 3, D-carbenicillin; 4, L-carbenicillin.

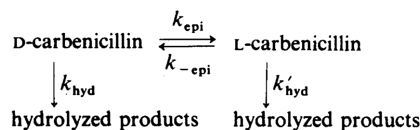
roetoposide, Nippon Kayaku Co., Tokyo, Japan; oxazepam, Banyu Pharmaceutical Co., Tokyo, Japan).

Preparation of Optically Active Oxazepam Acetate, Ethiazide and Carbenicillin Figure 1 shows the structures of the drugs studied. Etoposide and picroetoposide were optically pure, whereas the other drugs were racemates or epimeric mixtures. *d*- and *l*-Oxazepam acetate were prepared according to the previous paper.[12] Resolution of oxazepam acetate was carried out by HPLC on a chiral stationary phase (*N*-3,5-dinitrobenzoyl-(*R*)-phenylglycine phase, 4 × 250 mm, Sumika Chemical Analysis Service Ltd., Osaka, Japan). *d*- and *l*-Oxazepam acetate fractions were collected, respectively, and the solvent was removed under reduced pressure.

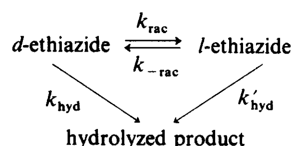
Each enantiomer of ethiazide was obtained by HPLC on the chiral stationary phase in a similar manner to oxazepam acetate. Ethiazide (2 mg/ml) was injected into a chromatograph (model 655A, Hitachi, Tokyo, Japan) and eluted with hexane–dichloromethane–methanol (20 : 20 : 1). *d*- and *l*-Ethiazide fractions were collected, respectively, and the solvent was removed under reduced pressure. Optically active ethiazide obtained was stable for one month at −15 °C.

D- and L-Carbenicillin were resolved by HPLC. Carbenicillin (10 mg/ml) was eluted with a mobile phase solvent (50 mM phosphate buffer, pH 7.0–methanol, 5 : 1) on a octadecylsilane bonded phase (TSKgel-ODS80TM, 4 × 150 mm, Tosho, Tokyo, Japan). Fractions of D- and L-carbenicillin were collected separately, and freeze-dried. Optically active carbenicillin obtained was stable for one month at −15 °C. Figure 2 shows typical chromatograms of D-, L-carbenicillin, and *d*, *l*-ethiazide samples obtained.

carbenicillin



ethiazide



etoposide

oxazepam acetate

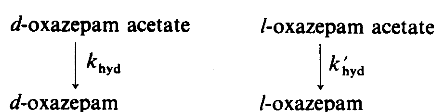


Chart 1

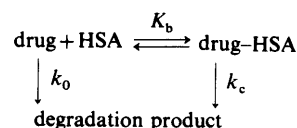


Chart 2

Kinetic Studies Apparent epimerization or racemization and hydrolysis rates of carbenicillin, ethiazide, etoposide, picroetoposide and oxazepam acetate were measured in the buffer solution at 37 °C and pH 7.4 (0.0667 M phosphate buffer, $\mu = 0.2$ adjusted with NaCl). The reaction was started by the addition of methanol solutions of ethiazide, etoposide and oxazepam acetate or aqueous solution of carbenicillin to HSA buffer solution. The final concentration of the drugs in the reaction mixture was 1×10^{-5} M for carbenicillin and ethiazide, and 5×10^{-6} M for etoposide and oxazepam acetate. The final concentration of methanol added was 2%. Aliquots of the reaction mixture were withdrawn at appropriate intervals and assayed for the drugs and their reaction products by HPLC under the conditions shown in Table I. The drug was extracted from the reaction solution into ethyl acetate (for ethiazide) or the mobile phase solvent (for oxazepam acetate), and subjected to HPLC.[12] For carbenicillin and etoposide, the reaction solutions were directly subjected to HPLC.

Estimation of Apparent Rate Constants in the Presence of HSA Apparent epimerization or racemization and hydrolysis rate constants of carbenicillin, ethiazide, etoposide, picroetoposide and oxazepam acetate were estimated from the time–concentration data of both isomers of each drug, according to Chart 1. A non-linear curve fitting program[22] was used for the estimation. Carbenicillin is epimerized at the 7 side chain (k_{epi} and k_{-epi}) and hydrolyzed at the β-lactam ring (k_{hyd} and k'_{hyd}) at neutral pH, as reported for moxalactam,[4] which is one of the oxacephem

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antibiotics and has a side chain structure similar to that of carbenicillin. It has been reported that benzothiazine diuretics such as ethiazide are racemized (k_{rac} and k_{-rac}),[5] and that ethiazide is hydrolyzed to the disulfonamide derivative (k_{hyd} and k'_{hyd}).[23] Reaction of etoposide can be represented by a consecutive reaction model, as reported in the previous paper.[8] Reverse epimerization of picroetoposide to etoposide and hydrolysis of etoposide to the *trans*-hydroxy acid derivative were not observed.

Estimation of the Binding Constant and Rate Constants of Bound Drug In the presence of HSA, the same reactions as represented in Chart 1 were observed for each drug, as described below. When the interaction between drug and HSA follows that in Chart 2, the apparent rate constants shown in Chart 1 can be represented by Eq. 1.[15]

$$k_{obs}=\frac{k_0+k_c\times K_b\times[\text{HSA}]}{1+K_b\times[\text{HSA}]} \tag{1}$$

where Drug–HSA is the Michaelis–Menten type complex between drug and HSA, K_b is the binding constant, and k_0 and k_c are rate constants of free and bound drug, respectively. [HSA] is the concentration of free HSA and can be approximated by the initial concentration of HSA, $[\text{HSA}]_0$, since $[\text{HSA}]_0$ is much larger than the initial concentration of drug.

The binding constant, K_b, and the rate constants for epimerization, racemization and hydrolysis of free and bound drug, k_0 and k_c, were estimated from the dependence of the apparent epimerization, racemization or hydrolysis rates on $[\text{HSA}]_0$ according to Eq. 1.

Determination of the Binding Constants Drug and HSA were incubated under the same conditions as those of the kinetic run. The drug which was not bound to HSA was separated by the ultrafiltration method (Micro Partition System-1, Amicon, Massachusetts, U.S.A.). The concentrations of free and total drug were determined by HPLC.

Results

Carbenicillin Figure 3 shows typical time-courses of the reaction of D-carbenicillin in the presence and absence of HSA. D-Carbenicillin was epimerized to L-carbenicillin and was also susceptible to hydrolysis at the β-lactam ring. The experimental data shown in Fig. 3 were fitted according to Chart 1 by using the non-linear curve-fitting program. Each isomer of carbenicillin was used as a starting material, and the rate constants estimated were similar for each isomer. The apparent epimerization and hydrolysis rate constants of D- and L-carbenicillin increased with increase in HSA concentration, and appeared to reach plateau values at high HSA concentrations. The binding constant, K_b and epimerization and hydrolysis rate constants of free and bound carbenicillin were estimated from the HSA concentration dependence of the apparent epimerization and hydrolysis rate constants of D- and L-carbenicillin according to Eq. 1 with the non-linear curve fitting program, and are summarized in Table II. Figure 4 shows the Lineweaver–Burk plots (Eq. 2) for epimerization and hydrolysis of D-carbenicillin in the presence of HSA. The straight lines shown in Fig. 4 suggest the formation of a Michaelis–Menten type complex between carbenicillin and HSA.

$$\frac{1}{k_{obs}-k_0}=\frac{1}{K_b(k_c-k_0)}\frac{1}{[\text{HSA}]_0}+\frac{1}{k_c-k_0} \tag{2}$$

The estimated value for binding constant, K_b, agreed with that determined by the ultrafiltration method. The epimerization and hydrolysis rate constants of free carbenicillin estimated from the data in the presence of HSA agreed with those estimated in the absence of HSA.

Ethiazide The thiadiazine ring of ethiazide has been reported to be hydrolyzed at neutral pH.[23] The time-courses of the reaction of *l*-ethiazide (Fig. 5) indicate that racemization of *l*-ethiazide to *d*-ethiazide also takes place. The apparent racemization and hydrolysis rate constants were estimated according to the model shown in Chart 1. In the presence of HSA, the reaction of etoposide could be represented by the same model. Each enantiomer of ethiazide was used as a starting material, and the rate constants estimated were similar. In the presence of HSA, racemization was retarded and hydrolysis was accelerated with increase in the HSA concentration. The racemization and hydrolysis rates appeared to reach constant values at high HSA concentrations. The binding constant, K_b, and the racemization and hydrolysis rate constants of free and

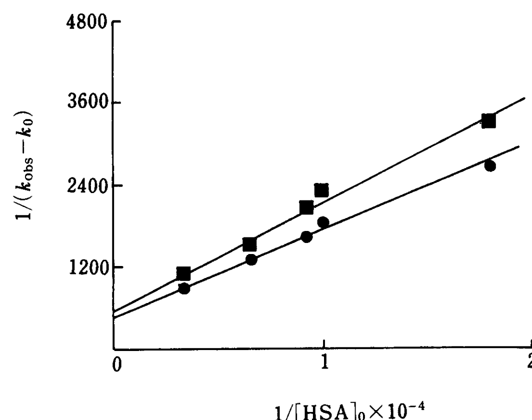

Fig. 4. Lineweaver–Burk Plots for Reaction of D-Carbenicillin with HSA.

●, epimerization; ■, hydrolysis.

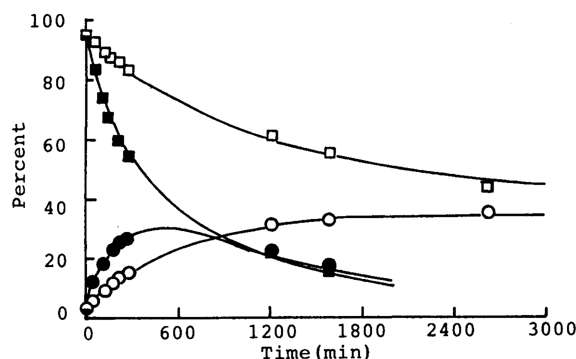

Fig. 3. Time-Courses of Reaction of D-Carbenicillin with and without HSA

□, ■, D-carbenicillin; ○, ●, L-carbenicillin; □, ○, without HSA; ■, ●, with 2.6×10^{-4} M HSA.

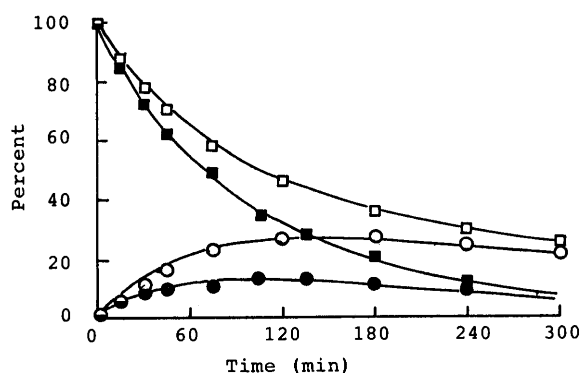

Fig. 5. Time-Courses of Reaction of *l*-Ethiazide with and without HSA

□, ■, *l*-ethiazide; ○, ■, *d*-ethiazide; □, ○, without HSA; ■, ●, with 2×10^{-4} M HSA.

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bound ethiazide were estimated by non-linear curve fitting according to Eq. 1, and are shown in Table II. The linear plots in the Lineweaver–Burk plot shown in Fig. 6 suggest that ethiazide and HSA form a Michaelis–Menten type complex. The binding constant for *l*-ethiazide estimated by the kinetic method was larger than that for *d*-ethiazide, whereas the binding constants determined by the ultrafiltration method were similar for *d*- and *l*-ethiazide.

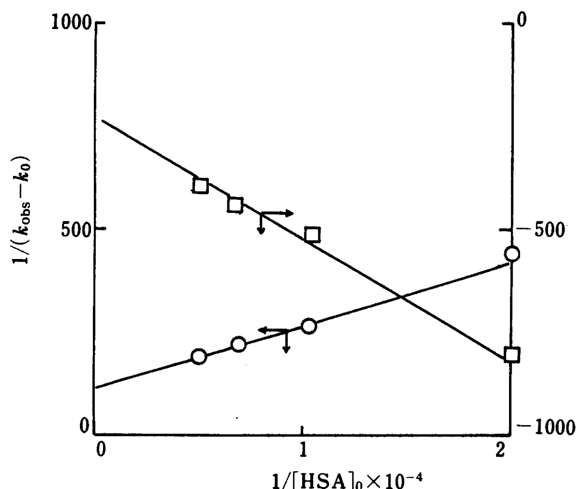

Fig. 6. Lineweaver–Burk Plots for Reaction of Ethiazide with HSA
□, racemization, ○, hydrolysis.

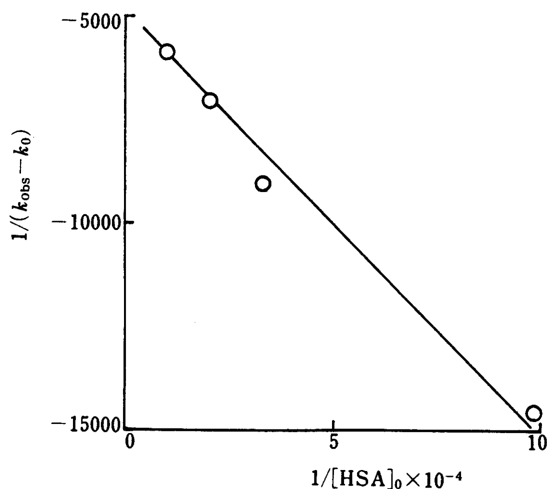

Fig. 7. Lineweaver–Burk Plot for Epimerization of Etoposide with HSA

Etoposide and Picroetoposide It has been reported that etoposide, having a trans-fused lactone in the molecule, is not hydrolyzed but epimerized to picroetoposide, which is an epimer of etoposide at position 2 (Fig. 1). The epimerization is irreversible and is followed by hydrolysis of the lactone ring of picroetoposide, as shown in Chart 1.[8] The epimerization rate of etoposide was decreased in the presence of HSA. The binding constant, K_b, and epimerization rate constants of free and bound etoposide were estimated from the dependence of the apparent epimerization of etoposide on HSA concentration according to Eq. 1, and are listed in Table II. The Lineweaver–Burk plot (Fig. 7) for epimerization of etoposide suggests that etoposide and HSA form a Michaelis–Menten type complex. The estimated value for K_b agreed with that determined by the ultrafiltration method. The epimerization rate constants of free etoposide estimated in the presence of HSA agreed with that estimated in the absence of HSA. The hydrolysis rate of picroetoposide was not affected by HSA. The binding constant for picroetoposide estimated by the ultrafiltration method is much smaller than that for etoposide. This indicates that picroetoposide is not bound to HSA at the highest concentration examined (1×10^{-4} M).

Oxazepam Acetate Oxazepam acetate has been reported to be hydrolyzed to oxazepam according to pseudo-first order kinetics in the pH range 1—10. Racemization of

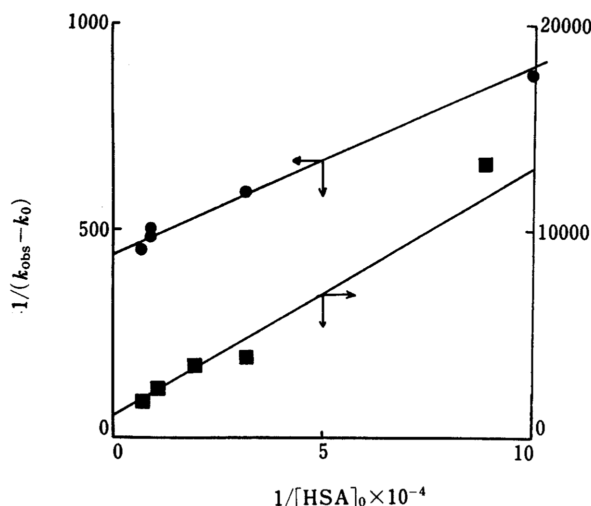

Fig. 8. Lineweaver–Burk Plots for Hydrolysis of *d*- and *l*-Oxazepam Acetate with HSA
●, *d*-oxazepam acetate, ■, *l*-oxazepam acetate.

Table II. The Binding Constant and the Degradation Rate Constants in the Presence of HSA at pH 7.4 and 37 °C

Drug	Binding constant × 10^{-3} (l/mol)	Rate constant × 10^4 (min^{-1}): Epimerization or racemization		Hydrolysis	
		k_c	k_0	k_c	k_0
D-Carbenicillin	3.80 (3.86)[a]	26.3	4.82 (4.88)[b]	16.7	0.576 (0.683)[b]
L-Carbenicillin	5.35 (5.99)	20.3	6.05 (5.70)	10.7	0.933 (1.03)
d-Ethiazide	7.81 (5.18)	15.3	58.8 (59.7)	114	27.1 (27.3)
l-Ethiazide	14.1 (5.80)	22.5	55.0 (56.2)	104	27.0 (27.8)
Etoposide	42.4 (40.0)	0.0878	2.12 (2.11)	N.D.	N.D.
Picroetoposide	N.D. (2.30)	N.D.	N.D.	N.D.	0.426 (0.426)
d-Oxazepam acetate	94.3 (80.6)	N.D.	N.D.	25.5	2.64 (2.58)
l-Oxazepam acetate	9.52 (12.5)	N.D.	N.D.	11.6	2.67 (2.58)

a) The binding constant estimated by using Micro Partition system-1. *b*) The rate constant estimated from the data obtained in the absence of HSA. N.D.: not detected. k_c and k_0 represent the degradation rate constants of free and bound drug, respectively.

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oxazepam acetate takes place as well as hydrolysis at alkaline pH.[12] In the presence of HSA at pH 7.4, the racemization of oxazepam acetate was not observed, whereas the hydrolysis of oxazepam acetate was accelerated by HSA. The binding constants for *d*- and *l*-oxazepam acetate and hydrolysis rate constants of free and bound oxazepam acetate were estimated from the dependence of the apparent hydrolysis rates on the HSA concentration according to Eq. 1, and are summarized in Table II. Formation of a Michaelis–Menten type complex between oxazepam acetate and HSA was suggested from the linear Lineweaver–Burk plots (Fig. 8) for hydrolysis of oxazepam acetate in the presence of HSA. The binding constant agreed with that determined by the ultrafiltration method. The hydrolysis rate constant of free *d*- or *l*-oxazepam acetate estimated in the presence of HSA agreed with that estimated in the absence of HSA. The hydrolysis rate constant of the *d*-enantiomer of oxazepam acetate bound to HSA was twice that of the *l*-enantiomer. This suggests that the esterase-like activity of HSA is enantioselective.

Discussion

The effects of HSA on the epimerization and racemization were complicated. In some cases HSA retarded the epimerization and racemization, and in other cases the rates were accelerated. The epimerization and racemization of the drugs examined in the presence of HSA could be represented by Chart 2. The epimerization of etoposide and the racemization of ethiazide were retarded by HSA. The binding of the drugs to HSA may inhibit the attack of hydroxy ion and/or water molecule and may retarded the epimerization and the racemization, as reported in the hydrolysis of meclofenoxate.[15] In the case of carbenicillin, however, binding to HSA accelerates epimerization and hydrolysis. Carbenicillin is negatively charged at the pH studied. Ion–ion and ion–dipole interactions between carbenicillin and HSA may activate the reaction center of carbenicillin favorably for the attack of hydroxy ion and/or water molecule.

Hydrolysis of carbenicillin, ethiazide and oxazepam acetate was accelerated by HSA. The hydrolysis rate of *d*-oxazepam acetate enantiomer bound to HSA was twice than that of the *l*-enantiomer, which suggests that the esterase-like activity of HSA is enantioselective. The different hydrolysis rates of oxazepam acetate enantiomers may be ascribed to the difference in the affinity of the drug enantiomers to HSA.

The epimerization rate of L-carbenicillin was larger than that of the D-isomer in the buffer solution, whereas D-carbenicillin bound to HSA was more rapidly epimerized than the L-isomer bound to HSA. The apparent epimerization rate of D-carbenicillin becomes larger than that of L-carbenicillin at HSA concentrations higher than about 2×10^{-4} M. *d*-Oxazepam acetate was hydrolyzed at a similar rate to the *l*-enantiomer in buffer solution, but *d*-oxazepam acetate bound to HSA was more rapidly hydrolyzed than the *l*-enantiomer bound to HSA (Table II). The apparent hydrolysis rate of *d*-oxazepam acetate was larger than that of *l*-oxazepam acetate in the presence of HSA. These results indicate that the reaction rates of chiral drugs in biological fluid are different from those in buffer solution. Differences in the pharmacokinetic properties of enantiomers, such as total body clearance[2] may therefore be ascribable to stereoselective degradation by HSA or enzyme in the body fluid. Chiral drugs which are epimerized/racemized and/or have labile ester or amide bonds would be especially susceptible to these effects.

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