Uptake and Stereoselective Binding of the Enantiomers of MK-927, a Potent Carbonic Anhydrase Inhibitor, by Human Erythrocytes *in Vitro*

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MK-927 [5,6-dihydro-4H-4(isobutylamino)thieno(2,3-B)thiopyran-2-sulfonamide-7.7 dioxide], a potent carbonic anhydrase inhibitor, contains a chiral center and exists as a racemate. In order to understand the kinetic behavior of the enantiomers of MK-927 in the body, the uptake and binding of these compounds were studied in human erythrocytes *in vitro*. Since no degradation or metabolism of the enantiomers occurred during incubation in blood, one can describe the equilibration of the drugs between plasma and erythrocytes by a closed two-compartment system. Erythrocytes were considered as a compartment composed of two parts: one in which free drug is exchangeable to plasma and the other in which drug is tightly bound to carbonic anhydrase in a Michaelis–Menten type binding. After the addition of the enantiomers individually to fresh blood, they were taken up by erythrocytes rapidly in a concentration-dependent manner. The time to achieve equilibrium decreased as the concentration increased, suggesting saturation of binding sites. With the assumption of simple diffusion, the binding and transfer kinetics were determined simultaneously by computer fitting. There were no stereoselective differences in the transfer process of the enantiomers across the erythrocyte membrane, while binding of the enantiomers exhibited stereoselectivity. The penetration of the unbound enantiomer across the erythrocyte cell membrane was rapid, with a mean transit time of about 3 sec. The *S*-(+)-enantiomer was bound to the high-affinity carbonic anhydrase isoenzyme more strongly than the *R*-(−)-enantiomer by approximately 10-fold. For the low-affinity isoenzyme, the *R*-(−)-enantiomer was bound more strongly than the *S*-(+)-enantiomer.

KEY WORDS: stereoselective binding; carbonic anhydrase inhibitor; enantiomers; MK-927; *in vitro*.

INTRODUCTION

MK-927 inhibits carbonic anhydrase in secretory cells of the ciliary process in the eyes to reduce aqueous humor production and intraocular pressure (1,2). It contains a single chiral center at the C_4 position of the thiopyran ring and thus exists as two enantiomers.

While present in most of the tissues, carbonic anhydrase predominates in red blood cells, accounting for more than 90% of the enzyme in the body (3). Earlier studies from this laboratory (4–6) have demonstrated that carbonic anhydrase, through its binding capacity, plays an important role in the elimination kinetics of the enantiomers of MK-927 and that the enzyme can be saturated when the drugs exceed the stoichiometric concentration of the enzyme.

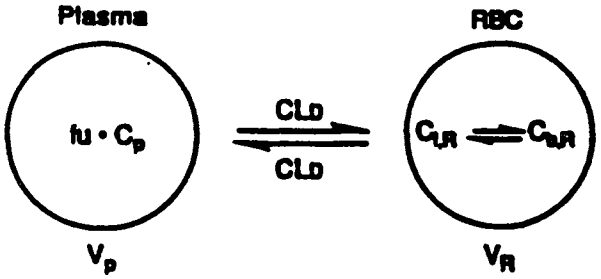
Unlike albumin and α_1-acid glycoprotein, carbonic anhydrase is present in erythrocytes rather than in plasma, and the erythrocyte cell membrane may serve as a significant diffusional barrier between the drug and the organs of elimination. It is, therefore, important to study the uptake and binding of MK-927 enantiomers by erythrocytes in order to better understand their kinetic behavior.

In this study, a closed two-compartment model was developed to relate the transfer process of MK-927 enantiomers across the erythrocyte membrane and the binding of these compounds to carbonic anhydrase in human erythrocytes. The transfer clearance and binding parameters of the enantiomers were simultaneously determined by computer fitting.

THEORETICAL

Kinetic Model

Since no degradation or metabolism of MK-927 enantiomers occurred during incubation in blood for 2 hr, it is reasonable to assume that the equilibration of the drugs between plasma and erythrocytes can be described by a closed two-compartment system (Model I). In this model, erythrocytes were considered as a compartment composed of two parts: one in which free drug is exchangeable to plasma, the other in which drug is tightly bound to two isoenzymes of carbonic anhydrase in a Michaelis–Menten type of binding.



Scheme I

Two isoenzymes of carbonic anhydrase showing different affinities for inhibitors have been reported to be present in human erythrocytes (7–9). Thus, the drug bound to erythrocytes ($C_{b,R}$) can be described by the following equation:

$$C_{b,R} = \frac{(np)_1 \cdot C_{f,R}}{K_{d_1} + C_{f,R}} + \frac{(np)_2 \cdot C_{f,R}}{K_{d_2} + C_{f,R}} \quad (1)$$

The subscripts 1 and 2 indicate the high- and low-affinity isoenzyme, respectively.

Total drug concentration in erythrocytes (C_R) can be expressed as the sum of bound ($C_{b,R}$) and unbound ($C_{f,R}$) drug concentrations.

$$C_R = C_{b,R} + C_{f,R} = \frac{(np)_1 \cdot C_{f,R}}{K_{d_1} + C_{f,R}} + \frac{(np)_2 \cdot C_{f,R}}{K_{d_2} + C_{f,R}} + C_{f,R} \quad (2)$$

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The differential equations for plasma and RBC compartments are

$$V_p \frac{dC_p}{dt} = -CL_D \cdot f_u \cdot C_p + CL_D \cdot C_{f,R} \quad (3)$$

$$V_R \frac{dC_R}{dt} = CL_D \cdot f_u \cdot C_p - CL_D \cdot C_{f,R} \quad (4)$$

Taking a derivative of C_R with respect to $C_{f,R}$ [Eq. (2)], we obtain

$$\frac{dC_R}{dC_{f,R}} = \frac{(np)_1 \cdot K_{d_1}}{(K_{d_1} + C_{f,R})^2} + \frac{(np)_2 K_{d_2}}{(K_{d_2} + C_{f,R})^2} + 1 \quad (5)$$

since

$$\frac{dC_R}{dt} = \frac{dC_R}{dC_{f,R}} \cdot \frac{dC_{f,R}}{dt} \quad (6)$$

Rearrangement of Eqs. (4), (5), and (6) yields

$$V_R \frac{dC_{f,R}}{dt} = \frac{CL_D \cdot f_u \cdot C_p - CL_D \cdot C_{f,R}}{\left[\frac{(np)_1 K_{d_1}}{(K_{d_1} + C_{f,R})^2} + \frac{(np)_2 K_{d_2}}{(K_{d_2} + C_{f,R})^2} + 1\right]} \quad (7)$$

where

C_p = drug concentration in plasma
C_R = drug concentration in erythrocytes
$C_{f,R}$ = unbound drug concentration in erythrocytes
$C_{b,R}$ = bound drug concentration in erythrocytes
V_p = volume of plasma (0.55 ml)
V_R = volume of erythrocytes (0.45 ml)
f_u = unbound fraction in plasma
CL_D = transfer clearance of unbound drug
K_{d_1} = dissociation constant for the high-affinity carbonic anhydrase isoenzyme
K_{d_2} = dissociation constant for the low-affinity carbonic anhydrase isoenzyme
$(np)_1$ = maximum binding capacity of the high-affinity isoenzyme
$(np)_2$ = maximum binding capacity of the low-affinity isoenzyme

Thus, CL_D, K_{d_1}, K_{d_2}, $(np)_1$, and $(np)_2$ can be determined by simultaneously fitting plasma concentration–time data to Eqs. (3) and (7) by using the Simusolv program (10).

Initial Estimates of Binding Parameters

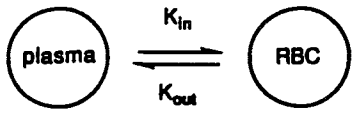
The initial estimates of binding parameters (K_d and np) were obtained by fitting binding data at equilibrium to the binding isotherm Eq. (1). The drug concentration in erythrocytes was calculated from the difference between plasma and blood concentrations corrected for the hematocrit value.

In addition, the drug bound to carbonic anhydrase ($C_{b,R}$) was estimated from the total drug in erythrocytes and free drug in plasma, assuming that (i) the free drug concentration in the cell water is equivalent to the free drug in plasma at equilibrium ($f_u \cdot C_p = C_{f,R}$) and (ii) the binding of the drugs to other components of erythrocytes is negligible.

The parameters K_d and np were estimated by computer fitting to Eq. (1) using the RS/1 program (BNN Software Products Company, Cambridge, MA).

Initial Estimates of Transfer Clearance (CL_D)

The equilibrium distribution of MK-927 enantiomers between plasma and erythrocytes can also be described by a closed two-compartment system, with K_{in} and K_{out} being the rate constants for the transport of total (bound + unbound) drug between plasma and erythrocytes (11).



Scheme II

Following the addition of the drug to the plasma compartments, changes in the amount of drug in plasma can be expressed by the following equation (11):

$$\left[A_t - \left(\frac{K_{out}}{K_{in} + K_{out}}\right) \cdot X_{total}\right] = \left[A_0 - \left(\frac{K_{out}}{K_{in} + K_{out}}\right) \cdot X_{total}\right] \cdot e^{-(K_{in} + K_{out}) \cdot t} \quad (8)$$

where A_0 and A_t are the amount of the drug in plasma at time 0 and time t, respectively. X_{total} is the total amount of the drug added to the system.

The term $e^{-(K_{in} + K_{out})t}$ will approach zero with time, and the amount of the drug in plasma (A_∞) reaches an asymptote.

$$A_\infty = \left(\frac{K_{out}}{K_{in} + K_{out}}\right) \cdot X_{total} \quad (9)$$

Substitution of A_∞ for $[K_{out}/(K_{in} + K_{out})] \cdot X_{total}$ to Eq. (8) and conversion to common logarithm yield

$$\log (A_t - A_\infty) = \log (A_0 - A_\infty) - \left[\frac{(K_{in} + K_{out})}{2.303}\right] t \quad (10)$$

Equation (10) states that a semilogarithm plot of $(A_t - A_\infty)$ versus time is linear, with a slope of $(K_{in} + K_{out})/2.303$.

Thus, the rate constants, K_{in} and K_{out}, can be estimated from the slope and Eq. (9). Since K_{in} is referred to as the total drug, the influx rate constant of unbound drug (K'_{in}) can be expressed as

$$K_{in}' = K_{in}/f_u \quad (11)$$

where f_u is the unbound fraction of the drug in plasma.

Thus, the transfer clearance of unbound drug (CL_D) can be estimated by the K'_{in} and plasma volume (V_p = 0.55 ml).

$$CL_D = K_{in}' \times V_p \quad (12)$$

MATERIALS AND METHODS

Materials

S-(+)- and *R*-(−)-enantiomers of MK-927 were synthesized at Merck Sharp & Dohme Research Laboratories (West Point, PA). The radiolabeled enantiomers were prepared with ^{14}C at C-1 of the isobutylamino group. Fresh whole blood samples from four drug-free normal healthy volunteers were collected from an antecubital vein into heparinized tubes. Experiments were conducted within 4 hr of collection.

Experimental Protocol

Uptake and Binding of ^{14}C-Enantiomers by Erythrocytes

Uptake and binding studies were performed *in vitro* by incubation of blood with drug at 37°C in a water bath at a rate of 80 oscillations/min. After preincubation of the blood for 5 min, the radiolabeled *S*-(+)- or *R*-(−)-enantiomer was added to the blood to yield a blood concentration range of about 2 to 200 μ*M*. Samples (1.0 ml) were taken at 10, 20, 30, 40, 60, 80, 120, 300, and 600 sec after drug addition and centrifuged immediately in an Eppendorf microcentrifuge (Brinkman, Centrifuge 3200) to separate the blood cells and plasma. The whole procedure was performed in less than 10 sec. The concentration of drug in plasma and whole blood was estimated from their radioactive equivalents.

Equilibrium between plasma and erythrocytes for either enantiomer was achieved within 2 min; thus, the mean concentrations of the drug at 2, 5, and 10 min were used for the estimation of binding parameters of the drugs. The drug concentration in erythrocytes was estimated from the difference between plasma and blood concentrations corrected for the hematocrit value.

Plasma Protein Binding of ^{14}C-Enantiomers

Binding of MK-927 enantiomers to plasma protein was determined by the filtration method (12). Briefly, the enantiomers were added to plasma to yield a final concentration of 0.1 to 50 μ*M*. After incubation at 37°C for 5 min, aliquots (0.8 ml) of plasma were transferred immediately to Centrifree tubes (Amicon Co., Danvers, MA) and centrifuged at 1500*g* for 5 min at 37°C. Under these conditions, approximately 150 μl of plasma filtrate was obtained. The unbound fraction of the drug was estimated from the ratio of drug concentration in plasma filtrate to the total drug concentration in the original plasma. Preliminary experiments revealed negligible binding to the filtration device (<1%).

Analytical Procedures

Radiohistograms and HPLC UV chromatograms of the incubated blood samples revealed that no degradation or metabolism of ^{14}C-enantiomers occurred in human blood during a 2-hr incubation at 37°C. Therefore, the radioactive equivalents are an accurate measurement of concentrations of MK-927 enantiomers in plasma and whole blood. Blood and plasma samples were combusted to $^{14}CO_2$ in a Packard Tricarb Sample Oxidizer (Model B306, Packard, Downes Grove, IL) and counted in a liquid scintillation counter, LKB-1219, Rack Beta (LKB, Wallace, Turku, Finland).

Statistical Analysis

Statistical analysis was determined by Student's paired *t* test; $P < 0.05$ was considered significant.

RESULTS

The enantiomers of MK-927 added to whole blood *in*

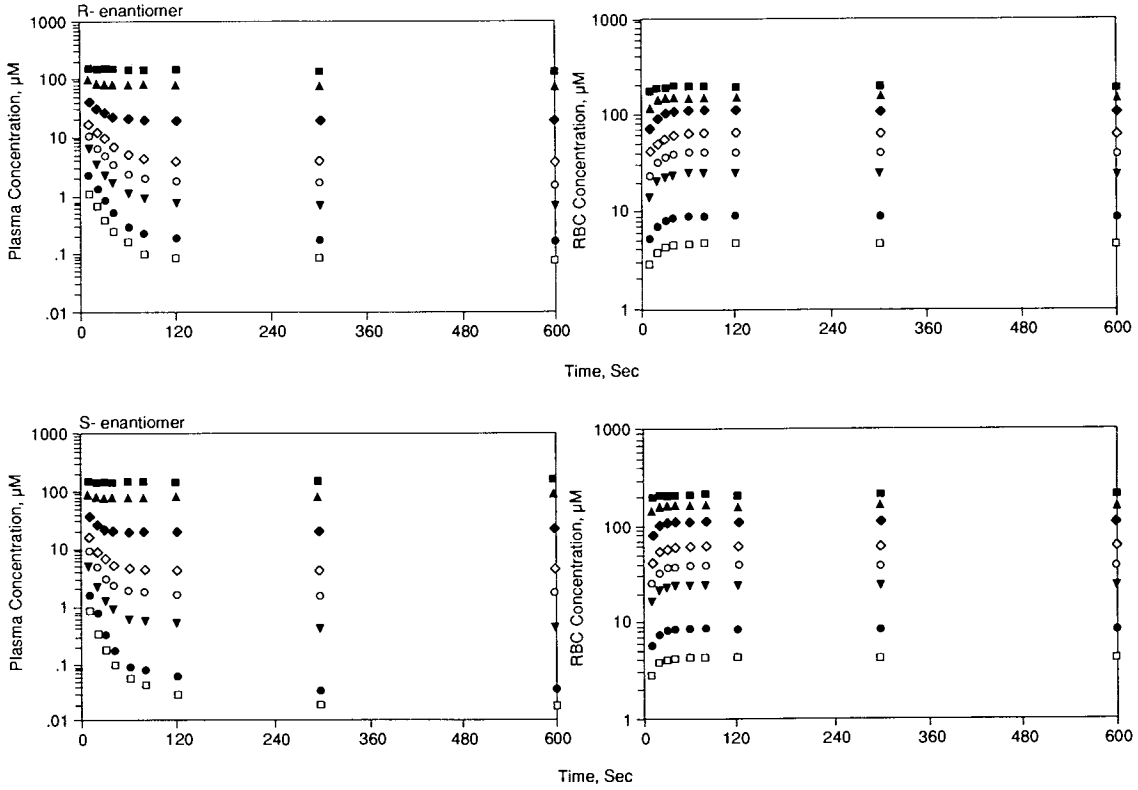


Fig. 1. Time courses of the concentrations of the *R*-(−)- and *S*-(+)-enantiomer in plasma (left) and erythrocytes (right) after addition of the drug to blood. Initial blood concentrations were 2 μ*M* (□), 4 μ*M* (●), 12 μ*M* (▼), 24 μ*M* (○), 48 μ*M* (◇), 72 μ*M* (◆), 144 μ*M* (△), and 200 μ*M* (■). The data plotted are from one of four subjects.

Table I. Unbound Fraction of the *S*-(+)- and *R*-(−)-Enantiomers of MK-927 in Human Plasma (Mean ± SD, $n = 4$)

Plasma concentration (μ*M*)	Unbound fraction	
	S-(+)-enantiomer	*R*-(−)-enantiomer
0.10	0.375 ± 0.042	0.261 ± 0.034
0.20	0.434 ± 0.040	0.269 ± 0.029
2.00	0.445 ± 0.042	0.251 ± 0.028
50.00	0.449 ± 0.043	0.245 ± 0.029
10.00	0.447 ± 0.045	0.266 ± 0.025
50.00	0.444 ± 0.042	0.261 ± 0.025

vitro were taken up very rapidly by erythrocytes in a concentration-dependent manner (Fig. 1). The time to achieve equilibrium decreased as the initial blood concentration increased; it took less than 10 sec (the first time point studied) to reach equilibrium at the highest concentration (200 μ*M*) and about 2 min at the lowest concentration (2 μ*M*).

The enantiomers were moderately bound to human plasma proteins, and the binding was independent of concentration over a wide range (Table I). The *R*-(−)-enantiomer appeared to be bound more strongly than the *S*-(+)-enantiomer; the mean unbound fractions for the *R*-(−)- and *S*-(+)-enantiomers were 0.258 and 0.432, respectively.

When the total concentrations of the enantiomers in RBC at equilibrium were plotted against their corresponding unbound concentrations in plasma, a curvilinear relationship was observed. However, a distinct plateau of total concentration of the drug in RBC was not reached (Fig. 2), suggesting that uptake of the enantiomers by erythrocytes was probably a sum of two processes: a linear simple diffusion process and saturable binding process presumably to carbonic anhydrase. With the assumption of simple diffusion, the concentration of bound drug in erythrocytes was calculated. The concentration of bound drug in erythrocytes reached a plateau of approximately 120 to 150 μ*M* (Fig. 2).

The binding parameters determined by fitting binding data at equilibrium to the binding isotherm equation [Eq. (1)] are summarized in Table II. For both enantiomers, there were two types of carbonic anhydrase in human erythrocytes: one had a high affinity but a low capacity, the other had a low affinity but a high capacity (Table II). The *S*-(+)-enantiomer was bound to the high-affinity isoenzyme more strongly than the *R*-(−)-enantiomer by approximately fivefold, whereas the *R*-(−)-enantiomer appeared to be bound more strongly than the *S*-(+)-enantiomer to the low-affinity isoenzyme, by about twofold. The binding capacity of the high- and low-affinity carbonic anhydrase isoenzymes was about 25 and 130 μ*M*, respectively (Table II).

Linear semilogarithmic plots of $(A_t - A_\infty)$ versus time [Eq. (10)] were observed only at the low blood concentrations (2 and 4 μ*M*), indicating that linear kinetics was valid only when drug concentrations were below 4 μ*M*. Thus, the influx rate constants (K_{in}) were determined at the concentrations of 2 and 4 μ*M* (Table III). The influx rate constants of unbound drug (K'_{in}) and transfer clearance are also listed in Table III. There were no stereoselective differences in the transfer parameters.

Using the initial estimates of binding parameters and transfer clearance, the K_{d_1}, K_{d_2}, $(np)_1$, $(np)_2$, and CL_D were determined simultaneously by fitting the pooled concentration–time data in Fig. 1 to differential Eqs. (3) and (7). The final fitting was deemed acceptable based on the standard deviation of the parameter estimates and the lack of systemic deviations in the residuals. The final estimates of binding and transfer parameters are listed in Table II. Figure 3 illustrates the best-fitted curves and the observed unbound plasma concentrations. In general, the fitting and observed data are in good agreement.

DISCUSSION

Erythrocytes as a diffusional "barrier" for hepatic drug elimination from blood and its implication in pharmacokinetics have recently been reported (13–15). It is important to determine whether erythrocytes are a diffusional barrier to the enantiomers of MK-927. The values of the transfer clearance (CL_D) for the enantiomers are about 10 ml/min (Table II). The mean transit time (MTT) of the enantiomers across the erythrocyte membrane is then estimated to be about 3 sec by the formula: $MTT = V_p/CL_D$ (where V_p is the plasma volume and $V_p = 0.55$ ml). This value is much less than the mean transit time of hepatic blood flow, approximately 10

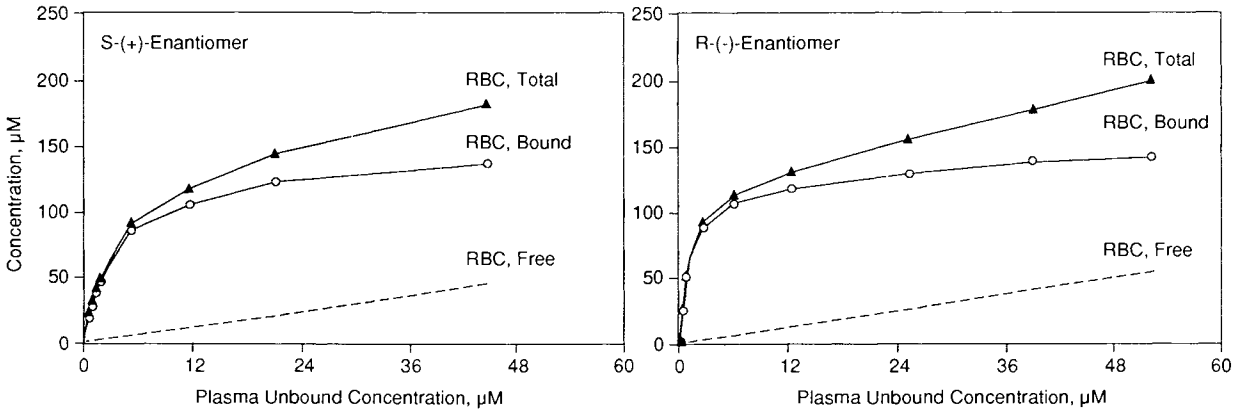


Fig. 2. Total, bound, and free concentrations of the *S*-(+)- and *R*-(−)-enantiomers in erythrocytes as a function of free drug concentration in plasma. Free drug concentration in erythrocytes (dashed lines) is assumed to be equal to free drug concentration in plasma.

Table II. Transfer and Binding Parameters of the Enantiomers of MK-927 in Human Erythrocytes (Mean ± SD, $n = 4$)

Enantiomer	CL_D (ml/min)	$(np)_1$ (μM)	K_{d_1} (μM)	$(np)_2$ (μM)	K_{d_2} (μM)
			Initial estimates[a]		
S-(+)	7.92 ± 0.88	27.4 ± 3.5	0.027 ± 0.006	142.2 ± 18.7	4.58 ± 0.50
R-(−)	8.75 ± 1.43	26.1 ± 1.8	0.138 ± 0.012*	123.7 ± 14.6	2.06 ± 0.027*
			Final estimates[b]		
S-(+)	9.08 ± 0.99	24.5 ± 4.8	0.023 ± 0.003	142.3 ± 15.7	3.66 ± 0.24
R-(−)	10.8 ± 1.6	30.1 ± 9.0	0.236 ± 0.049*	115.4 ± 24.9	2.41 ± 0.44**

[a] Initial binding parameters (K_d and np) were determined by computer fitting to Eq. (1) using the RS/1 program. Transfer clearance was obtained from Table III.
[b] Final estimates of CL_d, K_d, and np were determined simultaneously by computer fitting to Eqs. (3) and (7) using the Simusolv program.
* Significantly different from the *S*-(+)-enantiomer at $P < 0.001$.
** Significantly different from the *S*-(+)-enantiomer at $P < 0.01$.

sec (16), suggesting that the penetration of the unbound enantiomers across the membrane is not a rate-limiting step in drug elimination.

As indicated in Tables II and III, there were no stereoselective differences in the transfer parameters. Since there are no differences in lipid solubilities of the enantiomers, and since the transfer process of drugs across membrane is generally considered to be by passive diffusion, stereoselectivity in the transfer process of the enantiomers would not be expected. The transfer clearance (CL_D) values determined from the model with the pooled concentration–time data (Table II) were quite similar to that determined from partial plasma concentration–time data up to 2 min at 2 and 4 μM (Table III), indicating that the approach of obtaining the initial values was appropriate.

The time to achieve equilibrium was dependent on the blood concentration of the drug; it decreased as the concentration increased (Fig. 1). The concentration-dependent equilibration time may be explained by saturable binding of the enantiomers to carbonic anhydrase. Recently, Bayne and Hwang (17) presented a mathematical model to demonstrate the effects of nonlinear binding on equilibration time in a closed two-compartment system. They concluded that this was determined by a transfer rate constant as well as by the dissociation constant of binding and that the time to achieve equilibrium decreased as the binding was saturated. Our observations that the time to reach equilibrium decreased with increasing concentrations are consistent with their hypothesis.

Two isoenzymes of carbonic anhydrase showing different affinities for inhibitors have been reported to be present in human erythrocytes (7–9). Consistent with this, the binding data for the enantiomers of MK-927 also indicated two types of isoenzymes to which both enantiomers were extensively and stereoselectively bound (Table II). It is of interest to note that the stereoselectivity in binding was isoenzyme dependent. For the high-affinity isoenzyme, the *S*-(+)-enantiomer was bound more strongly than the *R*-(−)-enantiomer, while the reverse was true for the low-affinity isoenzyme (Table II). Binding to plasma was also stereoselective but to a lesser extent; the *R*-(−)-enantiomer was bound more strongly to plasma protein than the *S*-(+)-enantiomer (Table I). The similarity of the binding parameters determined by only the plasma data at equilibrium (Table II) and by the model with the pooled concentration–time data (Table II) indicated that the approach of obtaining the initial values of binding was appropriate.

The amino acid sequence and crystal structure of human carbonic anhydrase indicated that there is only one binding site per carbonic anhydrase molecule (18,19). Accordingly, the values of maximum binding capacities (np) for the high- and low-affinity carbonic anhydrase could be considered as being the concentrations of the isoenzymes. The concentrations of high- and low-affinity carbonic anhydrase in human

Table III. Influx Rate Constants and Transfer Clearance of the Enantiomers Across the Erythrocyte Membrane (Mean ± SD, $n = 4$)

Concentration (μM)	*S*-(+)-Enantiomer			*R*-(−)-Enantiomer		
	K_{in}[a] (min^{-1})	K_{in}'[b] (min^{-1})	CL_D[c] (ml/min)	K_{in}[a] (min^{-1})	K_{in}'[b] (min^{-1})	CL_D[c] (ml/min)
2	6.21 ± 0.68	14.4 ± 1.6	7.92 ± 0.88	4.11 ± 0.68	15.9 ± 2.6	8.75 ± 1.43
4	6.63 ± 0.79	15.3 ± 1.8	8.44 ± 0.99	4.08 ± 0.51	15.8 ± 1.9	8.69 ± 1.05
Statistical significance of difference	NS[d]	NS	NS	NS	NS	NS

[a] Influx rate constant of total drug.
[b] Influx rate constant of unbound drug; $K_{in}' = K_{in}/f_u$, where f_u is the unbound fraction of the drugs in plasma.
[c] Transfer clearance; $CL_D = K_{in}' \times V_p$ (where $V_p = 0.55$ ml).
[d] Not significant between 2 and 4 μM concentrations.

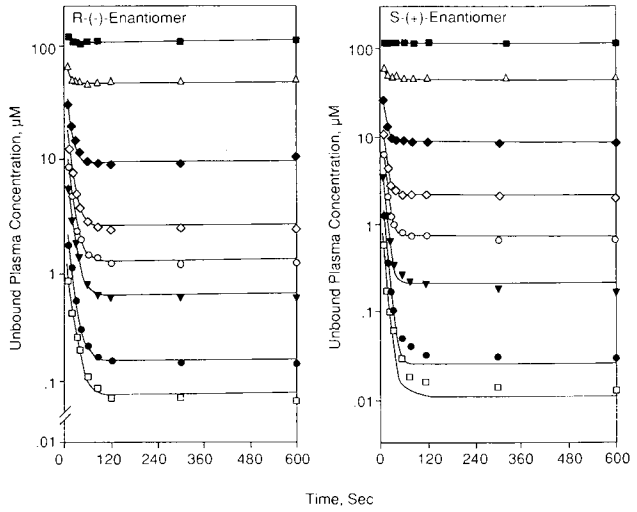


Fig. 3. Best-fit curves and the observed unbound concentrations of the *S*-(+)- and *R*-(−)-enantiomers in plasma after addition of the individual enantiomer to blood. Symbols as in Fig. 2.

erythrocytes were estimated to be 25 and 130 μ*M*, respectively (Table II). These values are in good agreement with those reported previously (20,21). The total carbonic anhydrase concentration in human erythrocytes estimated with acetazolamide was reported to be about 150 μ*M*, of which 20–30 μ*M* was the high-affinity isoenzyme and 120–130 μ*M* was the low-affinity isoenzyme (20).

In conclusion, there were no stereoselective differences in the transfer process of the enantiomer across the erythrocyte membrane. The penetration of the unbound enantiomers across the erythrocyte cell membrane was very rapid and not a rate-limiting step in drug elimination. On the other hand, binding of the enantiomers to carbonic anhydrase exhibited stereoselectivity. The stereoselective binding may play an important role in the disposition of the enantiomers *in vivo*.

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