Binding of Hydrochlorothiazide to Erythrocytes

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Hydrochlorothiazide (HCT) was administered orally to healthy volunteers and intravenously to rabbits. HCT concentrations in plasma (C_p) and erythrocytes (C_e) were determined by a high-performance liquid chromatographic method. C_e was about 9-fold that of C_p 24 h after the administration to volunteers, and 8-fold 6 h after the administration to rabbits. From the results of the *in vitro* binding study which was done with rabbit erythrocytes, at least the presence of three kinds of binding site for HCT was expected. The first binding site was characterized by extremely high affinity and very low capacity, and was unaffected by acetazolamide, known as a carbonic anhydrase inhibitor. The second one was characterized by medium affinity and medium capacity, and disappeared under the presence of acetazolamide and may be due to the carbonic anhydrase of erythrocytes. The third one was characterized by low affinity, but its binding capacity was extremely high and apparently unsaturable in the HCT concentration range studied (0.5–100 $\mu\text{g/ml}$ = 1.68–336 μM). The binding of HCT to erythrocytes seems to be dominated by the second binding site in the therapeutic range (under 1 $\mu\text{g/ml}$ of plasma).

Keywords — hydrochlorothiazide; diuretics; plasma concentration; erythrocyte concentration; carbonic anhydrase; erythrocyte binding; human; rabbit

Introduction

We have investigated the pharmacodynamics of hydrochlorothiazide (HCT) in human and animals^{1–7)} in order to clarify the relationship between the absorption behavior and the clinical response. In the previous study,³⁾ we reported that the HCT concentration in erythrocytes was about 10 times higher than in plasma in a nephrotic patient given HCT orally. We have attempted to characterize the partition of HCT to erythrocytes in this paper.

Materials and Methods

Materials — Powder and tablets (Esidrex®) of HCT were obtained from Ciba-Geigy (Japan) Ltd. All other reagents were of reagent grade.

Analytical Procedure — The concentrations of HCT in the samples were determined by the high-performance liquid chromatographic (HPLC) method described previously.³⁾

Clinical Study — A single dose of 100 mg of HCT (as 4 tablets of Esidrex®, 25 mg) was administered to 7 healthy volunteers (male,

22–27 years old, mean 24.1 years old; body weight 54–82 kg, mean 66.9 kg). Informed consent was obtained from the volunteers before study. Blood samples (2 ml) were taken from the forearm vein into a heparinized syringe, and centrifuged immediately to separate the plasma and erythrocytes.

***In Vivo* Study in Rabbits** — Male albino rabbits ($n = 3$), weighing 2.5–3.5 kg, were used. HCT in alkaline saline (pH 10–10.5 with NaOH) was injected into the marginal vein of one ear at a dose of 5 mg/kg. Blood samples were taken periodically from the contralateral ear vein.

Treatment of Erythrocytes — Fresh blood was drawn from rabbits, and immediately centrifuged (800 rpm, 10 min) to separate erythrocytes. The erythrocytes were then washed 5 times with 5 volumes of phosphate buffered saline (PBS). PBS (pH 7.4) consisted of 139.5 mM NaCl, 10 mM Na_2HPO_4 , 10 mM KH_2PO_4 and 1 mM CaCl_2 .

Time Course of the HCT Binding to Erythrocytes — The erythrocytes were suspended in PBS containing HCT at an hematocrit of

40.0% after washing. The concentrations of HCT in suspension were 1, 10 and 100 $\mu\text{g/ml}$. The erythrocyte density was determined by counting in a hemacytometer. The number of erythrocytes in the suspension was $(560 \pm 15) \times 10^4$ cells per mm^3 (cells per μl). The samples (1 ml) were incubated at 37 °C up to 4 h. After incubation the samples were centrifuged, and HCT concentrations in erythrocytes and medium were determined.

Effect of HCT Concentration in the Medium on the Binding of HCT to Erythrocytes — The procedure was just about the same way as for the time course study. The concentrations of HCT in the suspension were 0.5–100 $\mu\text{g/ml}$ ($= 1.68\text{--}336 \mu\text{M}$). The incubation was extended for 3 h. When the effect of inhibitor on the binding was studied, acetazolamide (AZ) was added to the suspension to give a 10-fold concentration of HCT.

Effect of Inhibitor on the Binding of HCT to Erythrocytes — HgCl_2 was added to the erythrocyte suspension in PBS at final concentrations of 5 and 10 μM . After 1 h incubation at 37 °C, the erythrocytes were washed twice with ice cooled PBS. Then HCT was added to the erythrocytes at a concentration of 5 $\mu\text{g/ml}$ ($= 16.7 \mu\text{M}$) and the samples were incubated for 2 h at 37 °C. In the case of AZ, HCT (16.7 μM) and AZ (1.67, 16.7 and 167 μM) were incubated with the erythrocyte suspension at 37 °C. Other experimental procedures are the same as for the

time course study.

Column Chromatographic Separation — The method as described by Dieterle⁸⁾ was followed. Hemolyzed erythrocyte was achieved by column-chromatography on Sephadex G-75 (2.2 i.d. $\times$ 93 cm). The mobile phase consisted of 25 mM phosphate buffer (pH 7.0) containing HCT (0.2 $\mu\text{g/ml}$). The ionic strength was adjusted to 0.2 with NaCl. Carbonic anhydrase activity was determined by the colorimetric method of Maren.⁹⁾ Hemoglobin and protein were monitored by measurement of absorption at 410¹⁰⁾ and 280 nm,¹¹⁾ respectively.

Results

HCT Concentration in Erythrocytes and Plasma Sample of Human and Rabbits

HCT concentrations in plasma and erythrocyte samples after oral administration to the healthy volunteers and intravenous administration to rabbits are shown in Fig. 1. In erythrocytes of volunteers, the peak concentration of HCT appeared somewhat later than in plasma, and was seen in 3 to 6 h. HCT concentration in erythrocytes was about 9-fold of that in plasma 24 h after the administration. The terminal half-lives of HCT in erythrocytes and plasma were 8.4 ± 1.2 and 5.4 ± 0.8 h, respectively.

In rabbits, (erythrocyte-to-plasma) ratio for HCT in 6 h after the administration was about 8.

Fig. 1. Decline of Hydrochlorothiazide Concentration in Plasma and Erythrocytes after Oral Administration (100 mg) to Volunteers ($n = 7$) and Intravenous Administration (2.5 mg/kg) to Rabbits ($n = 3$)

Each point represents the mean $\pm$ S.D. Key: ●, erythrocyte; ○, plasma.

Fig. 2. Decrease of Hydrochlorothiazide in the Incubation Medium by the Uptake by Erythrocytes

Each point represents the mean $\pm$ S.D. of four data. Initial concentration in erythrocyte suspension: 100 $\mu\text{g/ml}$ ($\circ$), 10 $\mu\text{g/ml}$ ($\bullet$), 1 $\mu\text{g/ml}$ (Δ).

The half-lives of HCT in erythrocytes and plasma were 2.2 ± 0.3 and 1.8 ± 0.3 h, respectively.

Binding Kinetics of HCT to Erythrocytes

The disappearance kinetics of HCT in the medium for 4 h are shown in Fig. 2. The kinetics showed similar monoexponential decline within 10 min. The disappearance rate constants for 100, 10 and 1 $\mu\text{g/ml}$ were 0.0805 ± 0.0052 , 0.0811 ± 0.0055 and 0.0817 ± 0.0060 min^{-1} , respectively. Equilibrium was attained in about 1 h. As shown in Fig. 2, HCT showed high affinity to erythrocytes, and the binding % by erythrocytes were 65, 75 and 88% at 100, 10 and 1 $\mu\text{g/ml}$ of initial HCT concentration, respectively.

Effect of Inhibitors on the Uptake of HCT

Preincubation of erythrocytes with HgCl_2 did

TABLE I. Effect of Inhibitors on the Uptake of Hydrochlorothiazide

Inhibitor	Concentration (μM)	Amount of hydrochlorothiazide in erythrocyte (%)
HgCl_2	0	100.0 ± 1.2
	5	102.2 ± 2.8
	10	97.6 ± 3.5
Acetazolamide	1.67	98.6 ± 2.9
	16.7	87.4 ± 2.3
	167	73.2 ± 5.3

Each value represents the mean $\pm$ S.D. of 5 experiments. The concentration of hydrochlorothiazide in suspension is 16.7 μM .

not cause any change in the uptake amount of HCT as shown in Table I. Also, no difference was observed on the preincubation with *N*-ethylmaleimide (data not shown). In the case of AZ, which is widely used as a carbonic anhydrase inhibitor, the binding ratio was decreased with an increase of AZ concentration in the medium.

Chromatographic Separation

Column chromatographic separation resulted in the elution profiles shown in Fig. 3. The peak of HCT was observed at the same retention volume as carbonic anhydrase. In this separation experiment, the column was eluted with mobile phase containing HCT to avoid dissociation of HCT from the HCT-receptor complex.⁸⁾

Binding of HCT to Erythrocytes

The binding of HCT to rabbit erythrocytes is shown as a Scatchard plot in Fig. 4. r_e is the number of moles of drug bound per erythrocyte, and $[D_f]$ is the concentration of free drug. The curve for HCT binding without AZ exhibited a curvature in $r_e/[D_f]$ with r_e and the plots seems to be on a nearly horizontal asymptote at higher r_e . The addition of AZ discharged the majority of the first phase observed at lower r_e , but the phase characterized by extremely high affinity and low capacity still remained.

Blanchard *et al.*¹²⁾ reported a three-parameter model to describe binding data involving multiple binding sites, which can be written as:

Fig. 3. Column Chromatography of Hemolyzed Erythrocytes from Rabbit Saturated with Hydrochlorothiazide
Key: —, carbonic anhydrase; ---, hydrochlorothiazide; ·····, hemoglobin; - · - · -, protein.

$$\frac{r}{[D_f]} = \frac{n_1 K_1}{1 + K_1 [D_f]} + C \quad (1)$$

$$r = [D_b]/[M] \quad (2)$$

where r is the number of moles of preservative bound per mole of macromolecule; $[D_f]$ and $[D_b]$ are the concentrations of free and bound drug, respectively; $[M]$ is the concentration of macromolecule; K_1 is the association constant; n_1 is the number of binding sites per macromolecule; and $C = n_2 K_2$.

The ratio (r) may also be expressed in other

dimensions,¹³⁾ such as moles (r_e) of drug bound per erythrocyte. When $[N_e]$ is the number of blood cell density, Eq. 1 may be written as:

$$\frac{r_e}{[D_f]} = \frac{[D_e]}{[D_f] [N_e]} = \frac{n_1 K_1}{1 + K_1 [D_f]} + C \quad (3)$$

where $[D_e]$ is the concentration of drug in erythrocytes; n_1 is the number of binding sites per erythrocyte (mol/cell). The parameters were calculated by use of the non-linear least-squares computer program MULTI¹⁴⁾ for the Eq. 3. n_1 , K_1 , and C were 1.18×10^{-18} mol/cell, 0.0214

Fig. 4. Scatchard Plot of the Binding of Hydrochlorothiazide with (○) and without (●) Acetazolamide
The broken lines are the least-square regression line obtained from Eq. 3 as 3-parameter model. The solid line is obtained from Eq. 4 as 5-parameter model.

$\times 10^9$ /cell, and 0.194×10^{-12} for HCT binding without AZ, and 0.119×10^{-18} mol/cell, 1.84×10^9 /cell, and 0.168×10^{-12} for HCT binding with AZ, respectively. The theoretical lines obtained from Eq. 3 were illustrated as broken lines in Fig. 4 by the method of Nørby.¹⁵⁾

From the curve for HCT binding with AZ, the presense of the binding site exhibiting extremely high binding affinity was observed. Thus, n_1 , K_1 and C obtained from the curve for HCT binding with AZ were substituted for Eq. 4 as a 5-parameter model and the plots for HCT binding without AZ were fitted for the equation which is written as:

$$\frac{r_e}{[D_f]} = \frac{n_1 K_1}{1 + K_1 [D_f]} + \frac{n_2 K_2}{1 + K_2 [D_f]} + C \quad (4)$$

The parameters obtained were as follow: K_1 , 1.84×10^{-9} mol/cell; K_2 , 0.158×10^{-6} mol/cell; n_1 , 0.119×10^{-18} mol/cell; n_2 , 3.21×10^{-18} mol/cell; C , 0.170×10^{-12} . The theoretical line obtained from Eq. 4 is illustrated as solid line in Fig. 4, and well fitted to the plots for HCT binding without AZ.

Discussion

From the results of the *in vitro* binding study to rabbit erythrocytes, shown in Fig. 4, the presence of three classes of binding site for HCT was expected. The first binding site characterized by extremely high affinity and very low capacity was unaffected by AZ which is known as a carbonic anhydrase inhibitor. The second one characterized by medium affinity and medium capacity disappeared under the presence of AZ and may be due to carbonic anhydrase of erythrocytes. This was supported by the facts that the second binding site disappeared by addition of AZ to the incubation medium and that the theoretical curves calculated from the 5-parameter model were well fitted to the data for HCT binding with or without AZ as shown in Fig. 4. The third one was characterized by low affinity, but its binding capacity was apparently unsaturable in the HCT concentration range studied ($0.5-100 \mu\text{g/ml} = 1.68-$

$336 \mu\text{M}$). In the studies of chromatographic separation, the only peak showing affinity to HCT was that of carbonic anhydrase. Thus, the first and third binding sites seem to be located in the membrane. Dieterle *et al.*¹²⁾ reported that chlorthalidone is preferentially taken up by erythrocytes and that the binding site is saturable for chlorthalidone. They identified the binding site as carbonic anhydrase. In our present study three classes of binding site were observed.

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