Binding of 2-Amino-4-chloro-*m*-benzenedisulfonamide as a Metabolite of Hydrochlorothiazide to Erythrocytes

Masaru YAMAZAKI,^{*,a} Soichi ITOH,^a Junko SAJI,^a Kazuhisa TANABE,^b Hajime NAKAHAMA,^c and Yoshimasa ORITA^c

Faculty of Pharmaceutical Sciences, Osaka University,^a Yamadaoka, Suita, Osaka 565, Japan and Department of Pharmacy^b and First Department of Internal Medicine,^c Osaka University Hospital, Fukushima, Fukushima-ku, Osaka 553, Japan. Received April 12, 1990

2-Amino-4-chloro-*m*-benzenedisulfonamide (ACBS) is a metabolite of hydrochlorothiazide. We reported that the ACBS concentration in erythrocytes was higher than in plasma in a patient. Therefore the binding of ACBS to rabbit erythrocyte was studied. The Scatchard plot showed the nonlinear plot and the horizontal asymptote. Curvature in this plot indicated the existence of 2 classes of binding. One class was at a specific site, probably at carbonic anhydrase. Chromatographic data seemed to support the possibility.

Keywords 2-amino-4-chloro-*m*-benzenedisulfonamide; hydrochlorothiazide; erythrocyte binding; rabbit; carbonic anhydrase; metabolite

Hydrochlorothiazide (HCT) has been used as a diuretic. Beermann *et al.* showed that 60—80% of HCT was absorbed in the upper gastrointestinal tract in healthy volunteers and it was excreted mainly in an unchanged form *via* the kidney after an oral dose.¹⁾ In 1987 we found 2-amino-4-chloro-*m*-benzenedisulfonamide (ACBS) in the urine, plasma and erythrocytes of patients who received HCT, and identified it as a metabolite of HCT.²⁾ After oral administration of HCT only 4.3% was converted to ACBS in urine collected for 24 h, and the concentration ratio of ACBS to HCT in plasma was 1 : 10 or less. However, the ACBS concentration in erythrocytes was about 20-fold higher than that in plasma. The present study was designed in order to research the affinity of ACBS for erythrocytes.

Materials and Methods

HCT was obtained from Ciba-Geigy (Japan) Ltd. Fresh blood was drawn from rabbits and immediately centrifuged. The erythrocytes were washed with phosphate buffered (pH 7.4) saline (PBS),³⁾ suspended in PBS containing ACBS (0.5—100 µg/ml = 1.75—350 µM) at an hematocrit of 40.0% (= (560 ± 15) × 10⁴ cells per mm³), and incubated at 37 °C for 3 h. When the effect of an inhibitor was studied, acetazolamide (AZ) was added to the suspension to give a 10-fold concentration of ACBS. The erythrocyte density was determined by counting in a hemacytometer. Other experimental procedures and analytical method were the same as for the previous study.²⁻⁴⁾

Results and Discussion

Blanchard *et al.*⁵⁾ reviewed the case of multiple classes of binding sites. When *m* classes of sites exist, each class *i* having *n_i* sites with a unique association constant *K_i*, can be written

$$\frac{r}{[D_f]} = \sum_{i=1}^{m-1} \frac{n_i K_i}{1 + K_i [D_f]} + n_m K_m \quad (1)$$

in which [D_f] is the concentration of free drug, and *r* is the number of moles of drug bound per macromolecule. Equation 1 ordinarily need not be extended beyond *m* = 3. The Scatchard plot yields a straight line when only one class of binding sites is present, and *r* is expressed in terms of two parameters.⁶⁾

The Scatchard plot for the binding of HCT to rabbit erythrocytes showed the nonlinear plot and the horizontal asymptote.³⁾ In this case, it was found that the data were fit by a five-parameter equation. By the addition of AZ (carbonic anhydrase inhibitor), the data were described by

the three-parameter model.³⁾ HCT was bound to three regions of the erythrocyte, the first class with a high affinity (*K*₁ = 1.84 × 10⁹ l/mol) and a low capacity (*n*₁ = 0.119 × 10⁻¹⁸ mol/cell), and the second class with a low affinity (*K*₂ = 0.158 × 10⁶ l/mol) and a large capacity (*n*₂ = 3.21 × 10⁻¹⁸ mol/cell). The third class was unsaturable binding. Our results suggested that the second class was at a specific site, probably at the carbonic anhydrase.³⁾

The binding of ACBS to rabbit erythrocytes was shown as a Scatchard plot in Fig. 1. Curvature in these plots indicated the existence of more than one class of binding. An attempt was made to fit the data in this study to two—five-parameter models; the data were best described by the three-parameter model.

$$\frac{r_e}{[D_f]} = \frac{n_1 K_1}{1 + K_1 [D_f]} + C \quad (2)$$

$$= \frac{(0.70 \times 10^{-18})(0.780 \times 10^6)}{1 + (0.780 \times 10^6)[D_f]} + 0.191 \times 10^{-12} \quad (3)$$

which was accomplished by use of a nonlinear least-squares computer program. *r_e* is the number of moles of drug bound per erythrocyte. The final term *C* is the horizontal asymptote on the Scatchard plot and accounts for the unsaturable class of binding sites. This model merely corresponded to a value of *m* = 2 in Eq. 1. The data gave the solid line

Fig. 1. Scatchard Plot of the Binding of 2-Amino-4-chloro-*m*-benzenedisulfonamide to Rabbit Erythrocytes with (○) and without (●) Acetazolamide

The solid line is obtained from Eq. 7 as a three-parameter model.

Fig. 2. Column Chromatography of Hemolyzed Rabbit Erythrocytes Saturated with 2-Amino-4-chloro-*m*-benzenedisulfonamide
—, carbonic anhydrase; —, 2-amino-4-chloro-*m*-benzenedisulfonamide; ····, hemoglobin; - - - - -, protein.

TABLE I. Binding of 2-Amino-4-chloro-*m*-benzenedisulfonamide and Hydrochlorothiazide by Rabbit Erythrocytes at 37°C, pH 7.4

Compound	K (l/mol)	n (mol/cell)	C
2-Amino-4-chloro- <i>m</i> -benzenedisulfonamide	0.780×10^6	0.70×10^{-18}	0.191×10^{-12}
Hydrochlorothiazide	0.158×10^6	3.21×10^{-18}	0.170×10^{-12}

shown in Fig. 1 when plotted according to Eq. 3.

In the presence of AZ $r_e/[D_f]$ was plotted against r_e , and then a linear relationship was observed as shown by the broken line in Fig. 1. The broken straight line ran parallel to the axis of abscissa, and was below the solid curve without AZ. However, the solid curve asymptotically approached the broken straight line in a high concentration of ACBS. From these observations, it would appear that the parameters of Eq. 3 involved the attraction of ACBS in the carbonic anhydrase region of the erythrocyte. The data of ACBS and HCT are summarized in Table I. The results, displayed in Table I, indicate that ACBS had 5-fold the affinity of HCT, but its binding capacity was one fifth of HCT.

In order to confirm the binding of ACBS to carbonic anhydrase, chromatography was done by the previous method.³⁾ Figure 2 illustrates the elution pattern of hemolyzed erythrocytes by affinity chromatography on Sephadex G-75, with phosphate buffer (pH 7.0) as an eluent. The peak of carbonic anhydrase coincided in position with the peak of ACBS.

Further studies should clarify the relationship between the transformable activity of patients and that of healthy persons.

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