petroleum ether–isoamyl alcohol is followed by a reextraction of the drug into aqueous sodium hydroxide, adjustment to pH 6.8 with acid, and extraction into chloroform for the final fluorometric measurement. With this procedure, percent recoveries of chlorzoxazone from plasma and urine compared to unextracted drug were 86.63 ± 1.66 (n = 8) and 95.37 ± 2.42 (n = 8), respectively. The linear working ranges for I in plasma and urine were 0.06–3.2 (r = 0.9993) and 0.13–3.0 (r = 0.9995) μg/ml, respectively, with minimum detectable levels at 60 and 130 ng/ml, respectively.

To demonstrate the application of the fluorometric I analysis, drug determinations in a commercial dosage form and in spiked plasma and urine samples were performed (Tables I and II). Plasma and urine data for spiked samples of I at 0.85, 3.4, and 6.8 μg/ml in the presence of varying concentrations of II and acetaminophen gave similar results. In addition, a plasma level–time profile for I (Fig. 1) illustrated the utility of the assay in determining plasma drug levels after oral administration of a single 300-mg dose to a healthy adult male. The peak plasma I level was obtained within 4 hr, with the drug essentially disappearing from plasma in 8 hr. Analysis of I in urine samples taken over 24 hr revealed that 116 μg of unchanged drug (<0.1% of administered dose) was excreted. These findings are comparable to those of previous metabolic studies (2).

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Acetazolamide Binding to Two Carbonic Anhydrase Isoenzymes in Human Erythrocytes

W. F. BAYNE, L.-C. CHU, and F. THEEUWES ˣ

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Abstract □ Acetazolamide binding to high activity and low activity carbonic anhydrase isoenzymes in red blood cells was studied. Inhibitory constants of 0.041 and 2.72 μ*M* and maximum binding capacities of 17.2 and 155 μ*M*, respectively, were found.

Keyphrases □ Acetazolamide—binding to two carbonic anhydrase isoenzymes, human erythrocytes, *in vitro* □ Carbonic anhydrase inhibitors—acetazolamide, binding to two carbonic anhydrase isoenzymes, human erythrocytes, *in vitro* □ Erythrocytes—acetazolamide binding to two carbonic anhydrase isoenzymes, *in vitro*

Acetazolamide, a carbonic anhydrase inhibitor widely used in the treatment of glaucoma, acts directly on the carbonic anhydrase in secretory cells of the ciliary body to reduce aqueous production and to lower intraocular pressure (1). The decrease in aqueous humor formation is thought to result from a decrease in bicarbonate flux from secretory cells (2, 3). Acetazolamide also acts indirectly to lower intraocular pressure by eliciting systemic acidosis (1). With the administration of therapeutic acetazolamide doses, renal carbonic anhydrase is inhibited, decreasing reabsorption of renally excreted bicarbonate (4).

BACKGROUND

Most carbonic anhydrase is in the red blood cells, with extravascular sources contributing an additional 10% or less (1). Two types of carbonic anhydrase have been identified: high activity type C and low activity type B (5). Extravascular carbonic anhydrase, including that found in the ciliary body, is generally the high activity type C isoenzyme (5). In humans, red blood cells are the major source of low activity type B isoenzyme (4). The ratio of low activity isoenzyme to high activity isoenzyme in human erythrocytes was reported as 6:1 (6).

Acetazolamide binding to human erythrocytes was studied previously (7). Intracellular and extracellular acetazolamide concentrations were determined by GLC following drug equilibration between the red blood cells and the external medium (either plasma or buffered saline). Rosenthal plots were constructed from the bound intracellular and extracellular concentrations to obtain the dissociation constants (inhibitory constant) of the enzyme–drug complex and the maximum binding capacity of the enzyme.

The inhibitory constant reported for the high activity isoenzyme was approximately an order of magnitude larger than that reported for the low activity isoenzyme (5, 6). Therefore, if *in vitro* binding studies are performed such that a wide range of intracellular acetazolamide concentrations is obtained, a definite curvature in the Rosenthal plot should be seen. Resolution of the curved plot into linear segments should permit the determination of maximum binding capacities and inhibitory constants for both isoenzymes.

Rosenthal plots constructed from *in vitro* equilibration studies performed in this laboratory exhibit a pronounced curvature. Inhibitory constants determined from the resolution of the curve were used as parameters in a nonlinear pharmacokinetic model for acetazolamide described previously (8).

EXPERIMENTAL

Human blood was drawn into heparinized tubes and immediately centrifuged to isolate the red blood cells. The cells were washed twice with two volumes of pH 7.4 buffered saline (0.15 *M* NaCl and 0.01 *M* phosphate), which were discarded. The cells were then suspended in the same buffered saline, and the hematocrit of the reconstituted red blood cell suspension was determined.

Acetazolamide was dissolved in methanol (0.5 μg/μl), and aliquots containing 1–200 μg of the drug were added to 20-ml disposable vials. Methanol was evaporated with a nitrogen stream. Red blood cell suspension was added to the vials (6 ml to vials containing <6 μg of acetazolamide and 3 ml to vials containing >6 μg of drug). The contents were incubated for 2 hr at 37° with gentle swirling in a reciprocating shaker. All incubations were performed using the suspension prepared from cells obtained from a single subject.

After incubation, the artificial blood medium was centrifuged. The acetazolamide concentration in 1–3-ml aliquots of the supernate was determined using a previously described high-pressure liquid chromatographic (HPLC) method (9).

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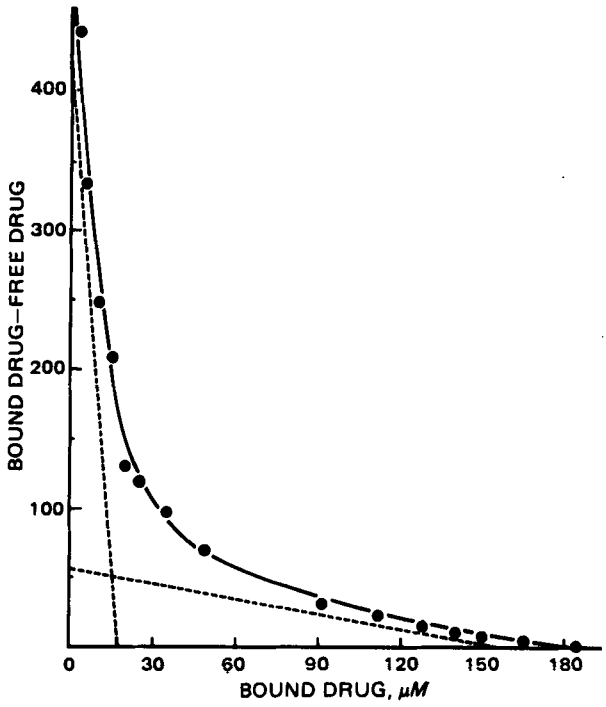

Figure 1—*Theoretical curve (—) that approximates the data points. The linear segment representing isoenzyme C has a negative reciprocal slope of 0.041 μM and an intercept on the abscissa of 17.2 μM; the linear segment representing isoenzyme B has a negative reciprocal slope of 2.71 μM and an intercept on the abscissa of 155 μM.*

The starting acetazolamide concentration in the artificial blood medium was assumed to be equivalent to the amount of acetazolamide added to each vial divided by the volume of the added suspension.

The total acetazolamide concentration in the red blood cells was calculated using:

$$C_{T,AB} = \frac{C_{AB} - (1-H)C_{ECF}}{H} \qquad \text{(Eq. 1)}$$

where C_{AB} is the concentration in the artificial blood, C_{ECF} is the concentration in the supernate or extracellular fluid, and H is the hematocrit.

RESULTS AND DISCUSSION

The acetazolamide concentration bound to carbonic anhydrase was calculated as the total intracellular concentration minus the product of the extracellular concentration times the cell water volume. The free drug concentration in the cell water was assumed to be equivalent to the extracellular concentration at equilibrium. The calculated bound drug concentration was expressed relative to the total cell volume, as was the measured total intracellular concentration.

A Rosenthal plot (Fig. 1) was constructed by plotting the ratio of the bound concentration to the intracellular free concentration *versus* the bound concentration. Except at bound concentrations that approached the total carbonic anhydrase concentration (intercept on abscissa), all intracellular drug essentially was bound (high bound to free ratio). The curvilinear plot was resolved into two linear segments by a graphic procedure (10). The negative reciprocal of the slope of each line is the inhibitory constant, and the intercept on the abscissa is the maximal binding capacity for the respective isoenzyme. The inhibitory constants were 0.041 and 2.72 μM and the maximum binding capacities were 17.2 and 155 μM for the high activity and low activity isoenzymes, respectively. Since there is one binding site per carbonic anhydrase molecule for the sulfonamides (11), the maximum binding capacity for a particular isoenzyme is the intracellular concentration of that isoenzyme.

The total carbonic anhydrase concentration in the human erythrocyte was reported to be 156 μM, of which 20 μM was the high activity isoenzyme and 136 μM was the low activity isoenzyme (6).

Previous studies (7) also showed that the carbonic anhydrase concentration (presumably type B) is variable in the human erythrocyte. Maren *et al.* (1) reported that the total carbonic anhydrase concentration is highly variable (range of 130–200 μM). Thus, the total carbonic anhydrase concentration in this study (172 μM) is within the range of previously reported values.

The inhibitory constant (2.72 μM) determined for the low activity isoenzyme in the present study agrees well with the inhibitory constant determined by previous investigators (7) (2 μM) using the *in vitro* equilibrium uptake technique. An inhibitory constant of 1 μM at 37° was reported for the low activity isoenzyme using kinetic measurements (6). The value reported at 37° in that study actually was extrapolated from measurements at lower temperatures.

Anions have been shown to inhibit carbonic anhydrase. For example, Maren *et al.* (5) showed that the low activity isoenzyme is much more sensitive to anion inhibition than the high activity isoenzyme (5). In particular, intracellular chloride and bicarbonate concentrations reduce the activity of the low activity isoenzyme to 8% of that in the absence of the anions. The intracellular chloride and bicarbonate anions probably compete with acetazolamide for binding sites in the uptake studies. Thus, the apparent inhibitory constant determined in studies may be larger than the true dissociation constant of the enzyme–drug complex in the absence of anions.

Intracellular red blood cell concentrations of chloride and bicarbonate are 85 and 15 mM, respectively (5). Chloride- and bicarbonate-ion concentrations that reduce the enzyme-catalyzed hydration of carbon dioxide by one-half (I_{50}) were reported to be 26 and 12 mM, respectively; these values approximate the inhibitory constants (5). Thus, the inhibitory constant of 2.72 μM, as determined in the present uptake study, becomes 0.18 μM in the absence of chloride and bicarbonate anions.

Maren *et al.* (1) reported an I_{50} value of 0.25 μM for acetazolamide using the low activity isoenzyme. These kinetic measurements were conducted using a barbital buffer to minimize carbonic anhydrase inhibition by anions in conventional buffer systems. Thus, there is agreement between the inhibitory constants determined by kinetic measurements and uptake studies if competition by intracellular anions is taken into account.

Since anions are present in the physiological situation, the inhibitory constants determined from uptake studies, rather than kinetic measurements, are more representative of the parameters to be used in describing drug disposition.

The I_{50} value reported for acetazolamide, using the high activity isoenzyme and the barbital buffer system, was 0.017 μM (1). This value compares favorably with the inhibitory constant (0.04 μM) determined from the uptake study. The similarity in these values is a manifestation of the negligible competition of intracellular anions with acetazolamide for the binding site on this isoenzyme. The negligible inhibition of the high activity isoenzyme by anions was demonstrated using kinetic measurements (5).

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